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Novel concepts for the treatment of HSV-2 and other enveloped viruses

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Abbreviations

%	percent
°C	degree(s)
µ	micro (10^{-6})
AIDS	acquired immunodeficiency syndrome
ANOVA	analysis of variance
ATP	adenosine triphosphate
AU	arbitrary units
BSA	bovine serum albumin
CCR5	C-C chemokine receptor type 5
CDC	Centers for Disease Control and Prevention
CPE	cytopathic effect
Đ	dispersity
Da	Dalton
DENV	Dengue virus
DMEM	Dulbecco's modified eagle medium
DMSO	dimethylsulfoxid
DNA	deoxyribonucleic acid
E	envelope protein of ZIKV
E. coli	Escherichia coli
EBOV	Ebola virus
eGFP	enhanced green fluorescent protein
ELISA	enzyme-linked immunosorbent assay
Env	envelope protein of HIV
ER	endoplasmatic reticulum
F	Trifluoridine
FACS	fluorescence-activated cell sorting
FcRn	neonatal Fc receptor
FCS	fetal calf serum
FITC	Fluorescein Isothiocyanate
g	gram(s)
g	gravitational force
gp	glycoprotein
GWUH	ZIKV isolate FB-GWUH-2016
h	hour(s)
HAdV5	human adenovirus type 5
HBV	hepatitis B virus
HCMV	human cytomegalovirus
HCV	hepatitis C virus
HEK293T	human embryonic kidney 293 large T antigen cells
HeLa	Henrietta Lacks
HFF	human foreskin fibroblasts
HIV	human immunodeficiency virus

HRP	horseradish peroxidase
HSV	herpes simplex virus
I	Idoxuridine
IC_{50}	half maximal inhibitory concentration
IgG	immunoglobulin G
k	kilo (10^3)
l	liter
lacZ	β-galactosidase gene
LB	lysogeny broth
LSM	laser scanning microscopy
m	meter
m	milli (10^{-3})
M	molar (mol/l)
MEM	minimal essential medium
MERS	Middle East Respiratory Syndrome
MeV	measles virus
MFI	mean fluorescence intensity
min	minute(s)
MN	number-average molar mass
MOI	multiplicity of infection
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
n	nano (10^{-9})
NADP(H)	nicotinamide adenine dinucleotide phosphate
NEAA	non-essential amino acids
PAA	poly(acrylic acid)
PAMPS	poly(2-acrylamido-2-methyl-1-propanesulfonic acid)
PAPA	poly((2-acrylamidoethyl)phosphonic acid)
PBS	phosphate buffered saline
PEAA	poly(ethylacrylic acid)
PFA	paraformaldehyde
pH	potentia hydrogenii
PMAA	poly(methacrylic acid)
PMEP	poly((2-methacrylamidoethyl)phosphate)
PMPA	poly((2-methacrylamidoethyl)phosphonic acid)
pp	pseudotyped particle
PPAA	poly(propylacrylic acid)
PSPA	poly(3-sulfopropyl acrylate)
PSVBS	poly(vinylbenzenesulfonate)
PVBzA	poly(vinylbenzoic acid)
PVPA	poly(vinylphosphonic acid)
PVSA	poly(vinylsulfonic acid)
RAFT	reversible addition fragmentation chain transfer
RLU/s	relative light units per second

RNA	ribonucleic acid
rpm	revolutions per minute
s	second(s)
SARS	severe acute respiratory syndrome
SEVI	Semen-derived Enhancer of Virus Infection
SOC	super optimal broth
STD	sexually transmitted disease
STI	sexually transmitted infection
Sup	supplementary
SV	Simian virus
T	thymidine
TAE	tris(hydroxymethyl)aminomethane base, acetic acid, EDTA
Tat	transactivator of transcription
$TCID_{50}$	tissue culture infectious dose 50
TK	thymidine kinase
TMB	3,3',5,5'-tetramethylbenzidine
TNA	therapeutic nucleic acid
Tris	trishydroxymethylaminomethane
UL	unique long
US	unique short
UV	ultraviolet
V	volt
v/v	volume per volume
VSV-G	vesicular stomatitis virus glycoprotein
w/v	weight per volume
WHO	World Health Organization
ZIKV	Zika virus
β-gal	β-galactosidase

One letter amino acid code:

A	Alanine	**I**	Isoleucine	**R**	Arginine
C	Cysteine	**K**	Lysine	**S**	Serine
D	Aspartic acid	**L**	Leucine	**T**	Threonine
E	Glutamic acid	**M**	Methionine	**V**	Valine
F	Phenylalanine	**N**	Asparagine	**W**	Tryptophan
G	Glycine	**P**	Proline	**Y**	Tyrosine
H	Histidine	**Q**	Glutamine		

1 Introduction

1.1 Viruses and antiviral therapy

Viruses are the most frequently occurring and versatile entities all over the world [50] with an estimated number of 10^{31} including viruses infecting animals and plants, as well as bacteriophages infecting bacteria and archaea [37]. Until 2018, 243 viruses were known that are able to infect humans [389]. This number increases by 4 new viruses each year, either by detecting already known viruses for the first time in humans or by discovering thus far unknown human infectious viruses [388]. The most common human pathogenic viruses and their main sites of replication in the human body [70,101] are summarized in Figure 1.

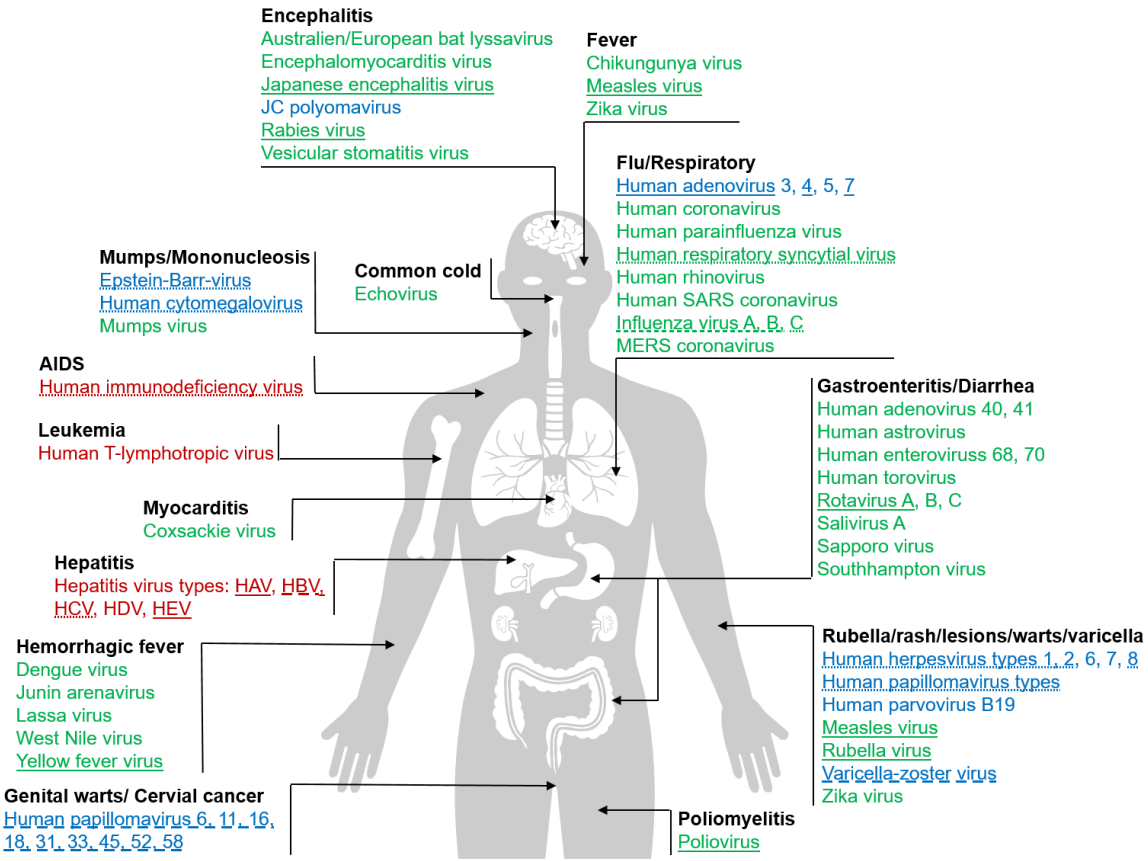

Figure 1: Most common human pathogenic viruses and associated symptoms or diseases .
The symptoms/diseases are written in bold and the main sites of replication of the causative viruses are indicated by arrows. Exception is Acquired Immunodeficiency Syndrome (AIDS) since it is a combination of complex symptoms. DNA viruses are shown in blue, RNA viruses in green and retroviruses in red. Viruses that can be treated with antiviral drugs are underlined with a dotted line. Viruses for which a vaccine exists are underlined with a continuous line. Viruses that are targeted by vaccines and by antiviral drugs are underlined with a dasehd line. MERS: Middle east respiratory syndrome; SARS: Severe acute respiratory syndrome.

One elegant opportunity to control viral diseases is to eliminate the causative agents or to prevent infection in the human population through active immunization using vaccines [7,18,32,370]. For some viruses, such as Hepatitis A virus, yellow fever virus, Japanese encephalitis virus or rabies virus vaccines already exist [148,257,336,344,354,384].

However, the genomes of viruses can be highly variable [34,158,207] and vaccination is relatively cost-intensive [32], which makes it necessary to develop antiviral drugs. Other viruses such as Hepatitis B virus (HBV), human papillomavirus (HPV), varicella zoster virus (VZV) and influenza viruses can either be prevented with vaccines or treated with antiviral drugs [70,225,350].

Unfortunately, antivirals can also be expensive [30,170,259,369,408] and their efficacy can be very low [145,172,230,341,374]. Furthermore, some viruses represent a huge socioeconomic burden by causing huge outbreaks [107,156,351,390] often favored by the development of resistances to existing drugs [66,229,263]. There is a pressing need to develop new antiviral agents to prevent outbreaks of viruses, such as severe acute respiratory syndrome (SARS) coronavirus in Asia between 2002 and 2003 [6,156] leading to 8456 infected people and 809 deaths [392] with estimated costs between 40 and 54 billion US dollars (USD) [177]. The flu pandemic caused by Influenza virus in 2009 [219,247] lead to 18,449 confirmed deaths [393] and between 151,700 and 575,400 people suffered the consequences of the infection [55,85]. Another outbreak was caused by Ebola in West Africa between 2013 and 2016 [149,390] with a total of 28,616 infections and 11,310 deaths in Guinea, Liberia and Sierra Leone [56,394] with costs around 53 billion USD [155]. Furthermore, the recent outbreak of the Zika virus in 2015, which is still ongoing [211,375] affecting 64 countries and territories [397] and costs between 7-18 billion USD in Latin America and the Caribbean [404].

As of 2016, only 22 infectious diseases can be treated either with vaccines and/or antiviral drugs [70]. In total, there are 90 antiviral drugs available, but only nine viruses out of the known 243 already discovered viruses can be treated [70,389]. These antivirals can be divided into 13 groups, which are summarized in table 1 [70].

Table 1: Summary of drug groups and the respective targeted viruses. Reviewed in De Clercq, E. and Li, G., Clin. Microbiol. Rev., 2016 [70]**.**

Drug group	Targeted virus
5-substituted 2-deoxyuridine analogues	HSV, VZV
Nucleoside analogues	HSV, VZV, HBV
(Nonnucleoside) pyrophosphate analogues	HCMV, HSV
Nucleoside reverse transcriptase (RT) inhibitors (NRTIs)	HIV, HBV
Nonnucleoside reverse transcriptase (RT) inhibitors (NNRTIs)	HIV-1
Protease inhibitors	HIV, HCV genotypes 1-4
Integrase inhibitors	HIV
Entry inhibitors	RSV, HSV, HIV, VZV
Acyclic guanosine analogues	HSV, VZV, HCMV
Acyclic nucleoside phosphonate analogues	HCV genotype 2 and 3
Hepatitis C virus (HCV) NS5A and NS5B inhibitors	HCV genotypes 1-6
Influenza virus inhibitors	Influenza viruses A, B and C, HCV, RSV
Immunostimulators, interferons, oligonucleotides and antimitotic inhibitors	HBV, HCV, HCMV, HPV related diseases

Although many antiviral drugs exist, most of them only combat the symptoms and may cause development of viral resistances due to the lack of broad-spectrum antiviral activity. Additionally, antivirals are often developed in the aftermath of a crisis. The overall goal would be to have treatments ready before an epidemic viral outbreak. Thus, new treatment strategies need to be developed to address these unmet medical needs [69,127,181,371].

1.2 Enveloped viruses

The so far known viruses that can infect humans are divided into 31 virus families including 18 enveloped and 13 non-enveloped viruses [102]. The viral envelope is a host cell derived lipid membrane, which is not present in non-enveloped viruses. They only have capsid proteins presented on their surface [358] to penetrate the cell membrane and to deliver the viral genome into the host cell [164]. Most of the viruses that are pathogenic to humans belong to the group of enveloped viruses [88], which is not surprising since the viral envelope is thought to have adapted to animal cells [121]. Furthermore, enveloped viruses are also able to spread from cell to cell [298] and to escape the adaptive immune response which might have resulted in the origin of a viral envelope [44,121]. The common mechanism that allows enveloped viruses to enter their target cells starts with their attachment through the interaction of viral glycoproteins with cellular surface receptors [73,97,117,328]. Subsequently, the interaction either triggers direct fusion of the viral and the cytoplasma membrane, which allows cytoplasmic delivery of the viral genome and proteins required for replication in the target cell [222,267,323]. Membrane fusion can also happen after the uptake of viral particles by macropinocytosis [4,365], phagocytosis [3,63] or receptor-mediated endocytosis [249,316].

1.2.1 Molecular biology of Herpes Simplex Virus Type 1 and 2

The family *Herpesviridae* consists of 90 identified virus species so far, omnipresent in the animal world and is grouped into three subfamilies, namely *Alpha-, Beta-* and *Gammaherpesvirinae* [173,208,283]. The genus *Simplexvirus* of the subfamily *Alphaherpesvirinae* includes two of the main human pathogenic species: Herpes simplex virus type 1 and 2 (HSV-1, HSV-2) [84].

HSV-1 and HSV-2 harbor a linear double-stranded (ds) deoxyribonucleic acid (DNA) of 152 kilo base pairs (kbp) in the electron-opaque core, which is enclosed by an icosahedral capsid [100,115,284,348,372] . This nucleocapsid consists of 12 pentameric and 150 hexameric capsomers and is surrounded by the tegument, an amorphous protein matrix [40,111,190,228,284]. The viral envelope is composed of parts of the host cell membrane which incorporates 600-750 viral glycoprotein spikes with varying lengths, spacing and angle tendency from the surface [9,25,100,136,241,329,335]. The average diameter of both viruses is 186 nm [136]. Although these two *Alphavirinae* members share a high sequence homology, they differ in their tropism, resulting in preferred replication in distinct anatomical sites and specific pathologies [1,118,123].

Initial infection routes of HSV are epithelial cells of the mucosa or skin [105]. The entry mechanism can be e.g. macropinocytosis, clathrin-mediated entry or fusion of viral and

cellular membranes [63,249,316]. The viral entry process of HSV can be divided into 3 parts: viral attachment and receptor interaction, membrane fusion and uncoating [241,402]. The initial step, the viral attachment is mediated by the contact between glycoprotein B (gB) and/or gC with cellular glycosaminoglycans, especially heparan sulfate [312,410]. After the viral attachment, the concertedly acting glycoproteins B, D, H and L (gB, gD, gH, gL) bind irreversibly to cellular receptors belonging to the class of Herpesvirus entry mediator (HVEM) [237], nectin-1 [71,122] and -2 [373] and 3-*O* sulfated heparan sulfate [311]. The next step, the fusion between viral and cellular membrane, is triggered by gD [116,140,187,330]. In the last phase after the membrane-fusion, the capsid is transported to the nuclear pore, the viral DNA is shuttled into the nucleus and three transcription rounds are initiated for viral replication [382].

Immediate early (IE or α), early (E or β) and late (L or γ) proteins are expressed afterwards [10]. IE proteins regulate the viral replication, E proteins are responsible for the synthesis and packaging of viral DNA and L proteins are mainly the structural virion proteins [294,296,372].

Both herpesviruses are neuroinvasive as well as neurotoxic and are able to establish lifelong latency [383]. After entering sensory neurons and retrograde transport to the cell nucleus, the viral DNA persists in an episomal (circular, non-integrated) form. The expression of the lytic gene is suppressed and the latency-associated transcripts (LAT) are expressed, leading to a latent state of the virus that is protected from the immune system [159,184]. Under specific circumstances the viruses can be reactivated and anterograde transported to the primary site of infection [159,184,383].

HSV-1 mainly causes oral herpes, also called herpes labialis, with characteristic painful orolabial blisters and ulcers [194,214,215,265,381] occurring 10 to 21 days after infection [380]. Transmission occurs through oral-to-oral contact allowing the endemic spread of HSV-1 [213–215]. In 2012, 3.7 billion people between 0-49 years were infected with HSV-1 worldwide [214,215]. While a decline of oral HSV-1 infections has been observed since the 1980s, the number of genital HSV-1 infections is rising. This can be explained by an increased popularity of oral-sex causing herpes genitalis [59,192,282], resulting in a total number of 140 million people worldwide living with genital HSV-1 infections in 2012 [214,215]. The highest prevalence of herpes genitalis is found in high-income countries such as America, Europe and Western Pacific [214,215] where HSV-1 infections in younger men who have sex with men as well as heterosexual women accounts for the majority of primary genital herpes [192,290].

Although the prevalence of herpes genitalis caused by HSV-1 infections is increasing, HSV-2 is still the predominant causative agent of herpes genitalis. HSV-2 is a sexually transmitted virus causing painful blisters and ulcers on the external genitalia [118,165,347] as well as dysuria [76,381]. Besides the symptoms of primary HSV-2 infection, reactivation of the virus can lead to inflammation of the genital tract and trigger symptoms such as fever, headache, malaise and myalgia [76,165]. Approximately 417 million people (~11%) between 15-49 years were infected with HSV-2 in 2012 [213,215]. HSV-2 seroprevalence

is up to 95% in female sex-workers and immunocompromised patients that are infected with the human immunodeficiency virus type 1 (HIV-1) [35,184,215].

Recently, it has been shown that the highest risk of transmission is during onset of the disease, but may also happen in the absence of any symptoms since most of the infections are asymptomatic [118,147,356]. Once the virus entered the human body, a lifelong infection due to latently infected neurons is established [26,175,295]. Under specific circumstances such as physical or emotional stress, fever, exposure to ultraviolet light or tissue damage, the virus can be reactivated from its latent state periodically, usually occurring at the site of primary infection [159,184,383]. They are milder, have a shorter duration compared to primary infections, and lack systemic symptoms [125,291]. In contrast, infections of neonates due to the contact of the fetus with HSV in the maternal genital tract is much more severe and can lead to neurologic disabilities or even death [12,16,110]. Furthermore, in immunocompromised patients, such as organ transplant recipients or HIV-1 infected persons, HSV infections comprise a significant cause of morbidity and mortality[206,265,292].

1.2.2 Treatment strategies of Herpes Simplex Virus Type 1 and 2 infections

The first approved antiviral drug against HSV was idoxuridine [167,270]. Idoxuridine belongs to the group of 5-substituted 2'-deoxyuridine analogues [68] and was approved by the Food and Drug Administration (FDA) in 1962 [167,271]. To be incorporated into the newly synthesized DNA it needs to be phosphorylated three times. One of the phosphorylation steps requires the viral thymidine which restricts the activation of the prodrug to infected cells [47]. During replication, the generated triphosphorylated idoxuridine is then falsely incorporated into the viral DNA [67,363]. This leads to strand termination and inactivation of the polymerase, which remains bound to the incomplete DNA chain [28].

Another nucleoside analogue, the acyclic guanosine analogue of deoxyguanosine, acyclovir, was approved by the FDA in 1981 [38]. Currently, treatment of HSV infections with acyclovir is still common and recommended by the World Health Organization (WHO), at least for herpes genitalis [391]. Unfortunately, the emergence of acyclovir-resistant viral variants is a common problem during long-term prophylaxis and treatment. In 95% of the cases the unique long UL23 gene coding for the viral thymidine kinase is affected [45,299]. As a consequence, the ability of the kinase to phosphorylate the nucleoside analogues is lost, or the substrate specificity is altered which renders these drugs ineffective [15,340]. The prevalence of resistances is 4- to 10-fold higher in immunocompromised compared to immunocompetent patients due to prolonged virus replication and a successful replication of less pathogenic viruses [15,265,266,340].

Increasing occurrence of drug-resistance, low bioavailability and the short half-life time of acyclovir fueled the development of newer nucleoside analogues such as valacyclovir, the l-valyl ester of acyclovir, penciclovir another acyclic guanosine analogue and famciclovir, the diacetyl ester of 6-deoxy penciclovir [21,27,265]. Unfortunately, some of them have

equivalent modes of action, allowing the development of cross-resistances or unwanted adverse effects occurred [27,352].

Since most of the mutations leading to resistances occur in the viral thymidine kinase, further treatment alternatives include substrates for the viral DNA polymerase, such as the acyclic nucleoside phosphonate derivatives cidofovir and adefovir. Cellular kinases mediate two phosphorylation steps of the substrates, increasing their affinity for viral DNA polymerases compared to cellular polymerases [27,265]. Another alternative to overcome low bioavailability is the administration of foscarnet. It is a pyrophosphate analogue which directly binds to pyrophosphate-binding site of the viral DNA polymerase and inhibits the DNA elongation, but is exclusively used to treat immunocompromised patients due to its nephrotoxicity [27,265,381].

Although current treatment regimens for HSV infections reduce the severity and duration of infection, prevent the transmission, suppress the recurrence and promote the healing of the site of infection, they are not able to eliminate the virus from the host [27,105,265]. In addition to the challenging issues that HSV establishes latency and is reactivated at certain occasions [159,184,339,383], another important challenge is the treatment of immunocompromised patients [142,206,305]. There is still no vaccine available to prevent a HSV infection, but several candidates are in clinical phase II trials [165]. To date, the best opportunity to prevent HSV infections is the avoidance of oral and unprotected sexual contact and sharing objects such as drinking bottles with suspected HSV patients [105,118,367].

1.2.3 Molecular biology of Zika virus

The family *Flaviviridae* comprises of over 50 species of arthropod-borne viruses. Zika virus (ZIKV), which caused an epidemic outbreak in 2015 is one of the representatives of the *Flaviviridae* and closely related to other human pathogens such as Dengue, West Nile, Japanese encephalitis and Yellow fever virus [318]. ZIKV was first isolated in 1947 from a sentinel rhesus macaque in the Zika forest in Uganda [87]. Between 1947 and 2007 ZIKV was present as a sylvatically cycling pathogen in Africa and Asia. Infrequent infections of human, accompanied by fever-like illness or without any symptoms, were noticed [22,106,317].

ZIKV belongs to the group of positive sense single-stranded (ss) ribonucleic acid (RNA) viruses. The virus has a diameter of around 50 nm [319]. The genome has a size of approximately 10.8 kb and encodes for three structural and seven non-structural (NS) proteins [198,319]. The non-structural proteins contribute to various replication steps such as transcription, polyprotein processing, virus assembly and host immune response antagonism [319]. NS3 shows homology to helicases and NS5, the largest viral protein with 103 kDa that has two functions by acting as a RNA-dependent RNA polymerase and capping of RNA [182,400,418,421]. Structural proteins include the capsid (C), the membrane (M) and the envelope (E) [319]. The RNA is encased by the capsid and after its translocation into the cytoplasm it is translated into a polyprotein. It is thought that the polyprotein is then cleaved, processed and the RNA is then synthesized by the above mentioned non-structural

proteins [57,103,107]. Virus particles are assembled, and bud into the lumen of the endoplasmatic reticulum (ER). After their final release by exocytosis, the viral particles carry a host-cell derived membrane [176]. An icosahedral shaped envelope covers the membrane with 180 glycoproteins with lipid-membrane anchored E and M proteins. The dimeric E protein is mainly present at the surface of the lipid-membrane and is thought to be the mediator of viral attachment and membrane fusion [318].

The cell tropism of ZIKV is broad and dermal fibroblasts, epidermal keratinocytes, neural progenitor cells and embryonic stem cells can be entered [139,346]. This is mainly due to the interaction with many broadly expressed host cell molecules such as glycosaminoglycans (GAGs) or phosphatidylserine receptors of the TIM (T-cell immunoglobulin and mucin domain) and TAM (Tyro3, Axl, and Mer) families [139].

The transmission of ZIKV is mainly through bites of the mosquito species *Aedes aegypti* and *Aedes albopictus*, which are common in (sub-) tropical regions such as Asia and the Americas as well as distinct areas in Africa and Europe with close proximity to humans [150,293]. In 2007, the first ZIKV outbreak outside Asia and Africa on Yap Islands in Micronesia was reported [93]. The next large outbreak occurred in French Polynesia, affecting 66% of the population in 2013 [49] and in Brazil with 1.3 million infected people in 2015 [395]. ZIKV became an epidemic infection in 2015 and as of March 2017 64 countries or territories reported mainly vector-borne ZIKV transmission [398]. The spread of the Zika virus is shown in Figure 2.

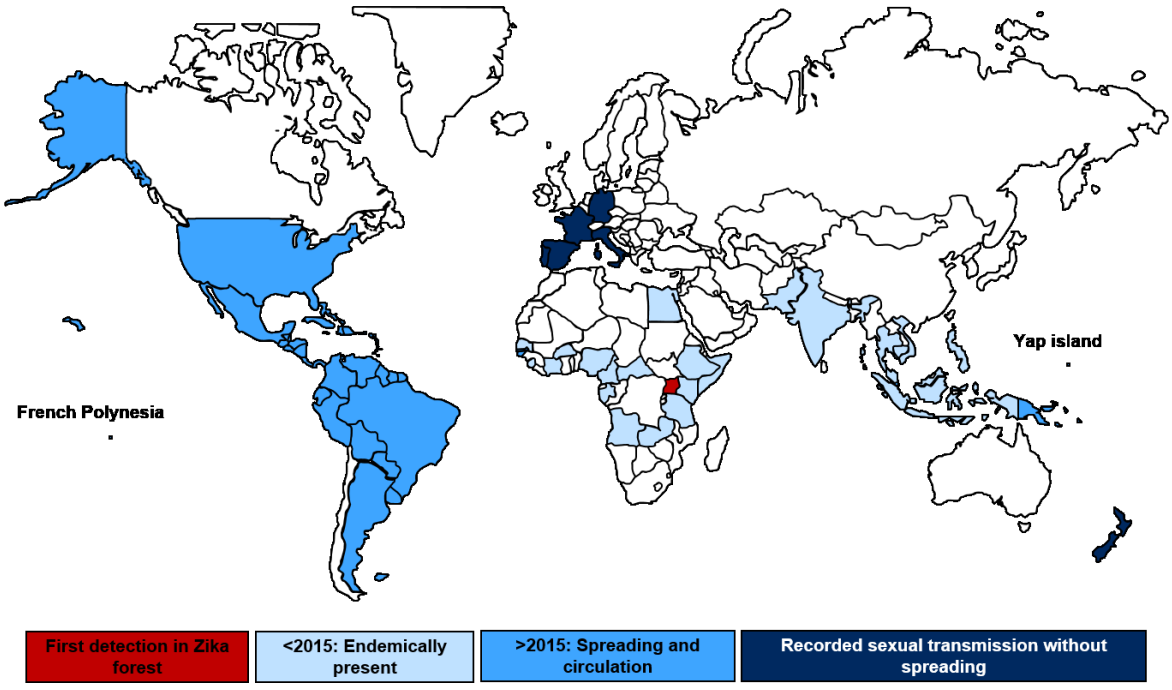

Figure 2: Zika virus distribution indicated on a global map between 1947 and 2017.
ZIKV has been first detected in Uganda in 1947 (red). Before 2015 it was endemic in Africa and Asia (light blue). After 2015 it spread east and circled the globe (blue). Some countries reported sexual transmissions but ZIKV is not actively spreading (dark blue). Data obtained from WHO (ref).

In 20% of the adults ZIKV infection causes Zika fever or Zika virus disease, a fever and/or rash which is accompanied by arthritis, arthralgia and/or conjunctivitis [93,317,412]. Since

ZIKV has a broad cell tropism involving also neuronal cells, occasional cases of meningoencephalitis and myelitis occur [52,231]. The neurotropic potential manifests in ZIKV-associated Guillain-Barré syndrome as wells as microcephaly in newborns. Guillain-Barré syndrome is an acute peripheral neuropathy caused by the demyelination of peripheral nerves by the host immune system [39,49,250]. Microcephaly is characterized by reduced head size and impaired development of fetal brain, as well as further birth defects including intracranial calcifications, visual and audible impairment, intrauterine growth restriction or even fetal demise [78,387]. These birth defects and the overall adverse pregnancy outcome, regardless of the trimester in which the infection occurred, forced the WHO to declare a Public Health Emergency of International Concern for ZIKV epidemic areas at the beginning of 2016 [275].

Since ZIKV can also cross the placental barrier, vertical transmission from mother to child in possible [79]. The virus has been detected in amniotic fluid, placenta and the fetal brain [48,91,224]. Further ZIKV containing body fluids, are saliva [19], breast milk [95] and semen [135]. Since several cases of male-to-female transmission following unprotected vaginal intercourse have been reported, sexual transmission also seems to be a possible transmission route [82,135,240].

1.2.4 Treatment strategy of Zika virus infection

Until now, there is neither a vaccine nor a drug available for the treatment of ZIKV infections. The best way to prevent ZIKV infection is the avoidance of mosquito bites. Since it is assumed that ZIKV is sexually transmitted the use of condoms in high-risk regions is recommended by the WHO to prevent the spreading of this disease. Persons with a confirmed ZIKV infection, pregnant women or people who have traveled to areas with a high ZIKV prevalence should also perform safer sex or stay abstinent for at least for 6 months [251,396].

1.3 Broad-spectrum antiviral agents

The best way to fight pathogenic viruses, would be to find a universally acting antiviral agent [86]. For bacteria it is common practice to use broad-spectrum antibiotics to treat more than one bacterium with a drug [24]. Such a “one drug, multiple-bug” approach has already successfully been used for lubricants of condoms marketed as Dual Protect^{TM} for additional prevention of infection by sexual transmitted viruses such as human immunodeficiency virus (HIV-1) and herpes simplex virus type 2 (HSV-2) [72,289,337]. Such microbicides can exhibit a broad-spectrum of antiviral activity by targeting the first crucial step that all viruses have in common: entry into the host target cell [154]. There are already some ways described to inhibit the viral entry process. One opportunity is to alter the physical chemistry of viral membranes since repairing mechanisms are not accessible after the viral budding from the host cell membrane [385]. This was realized for HIV-1 with a cholesterol interacting and sequestering peptide leading to a permeabilization of the virus [51]. Since cholesterol is present in many viruses as a result of budding from the host cell membrane [154,253,342,401,407] it represents a target for broad-spectrum antiviral activity [51]. Other

peptides that mimic parts of the viral fusion protein and thereby interacting with cellular receptors might also be useful broad-spectrum antivirals, at least for the same virus family, such as paramyxoviruses [227]. Furthermore, a small indole-derivative molecule named Arbidol is able to inhibit several enveloped and non-enveloped viruses (HBV, HCV, chikungunya virus or coxsackie virus) due to the interaction with the polar head of phospholipid membranes and aromatic amino acid residues [31]. Rigid amphipathic fusion inhibitors (RAFI) act as membrane-binding photosensitizers against HSV-1, HSV-2, HCV and vesicular stomatitis virus. Under light exposure, singlet oxygen ($^1O_2$) is generated in the membrane, which leads to a lipid oxidation and therefore an increased membrane rigidity. The biophysical change of the viral envelope inhibits the fusion with the host cell [8,331,333,364].

Another approach is the inhibition of the viral genome replication. A small-molecular-weight compounds (A3) has been found to block the *de novo* pyrimidine synthesis of uracil monophosphate and thereby inhibiting various viruses such as influenza virus A and B, vesicular stomatitis virus, HCV, West Nile virus, dengue virus and HIV-1 [146]. Other molecules, such as Ribavirin interact with the purine pathway. Ribavirin is a nucleoside analogue and is able to inhibit viral replication of HCV, RSV and influenza virus [14,256,315]. Recent studies showed that Ribavirin is also active against Marburg virus [232], norovirus [179] and Chikungunya virus [168].

Other broad-spectrum antivirals belonging to the group of thiazolides are concentrated on host-regulated processes such as blocking the maturation of hemagglutinin in influenza virus [286], inhibition of glycoprotein formation of rotavirus [113], activation of genes that block viral replication of HCV and HBV [285,287], dengue virus and yellow fever virus [234] as well as Japanese encephalitis virus [309].

Polymers with an anionic charge, have also been described for their antiviral effects. Their potential as broad-spectrum antiviral agents were already demonstrated in the 1960's. Polymethacrylic acid (PMAA) was the first polymeric anion shown to inhibit the infection of vesicular stomatitis, sindbis and vaccinia viruses [326,327]. Especially sulphated polymers, polyphosphates, nucleic acid polymers or carbosilane dendrimers were able to inhibit infection with enveloped viruses such as HIV-1, HSV-1/-2 and influenza virus [188,216,306]. They inhibit the fusion of viruses with cells either by interacting with viral glycoproteins [306,361] or by binding to cellular receptors [65,306]. So far, a specific structure-activity relationship of such charged polymers is missing, but is essential for the efficient design and development of new broad-spectrum antiviral agents [216].

1.4 Albumin-based drug delivery

Many drugs have a short half-life because of their fast clearance by the kidneys or the liver. In recent years various strategies have been evolved to optimize the pharmacokinetics of drugs e.g. by the conjugation with albumin [23,151,197,243,274,411,414]. Human serum albumin has a high abundance in plasma, a well-defined structure, a long half-life of 19 to 21 days in the human body [261,262] and excellent binding properties for endogenous and exogenous compounds [414]. These properties make albumin a favorable candidate for the

improvement of drug delivery. The long half-life is a result of the recognition by the neonatal Fc receptor (FcRn), which is expressed in many cell types as well as organs and mediates the recycling of albumin back into the bloodstream following cellular uptake. The recycling pathway is illustrated in Figure 3 [178,303,325].

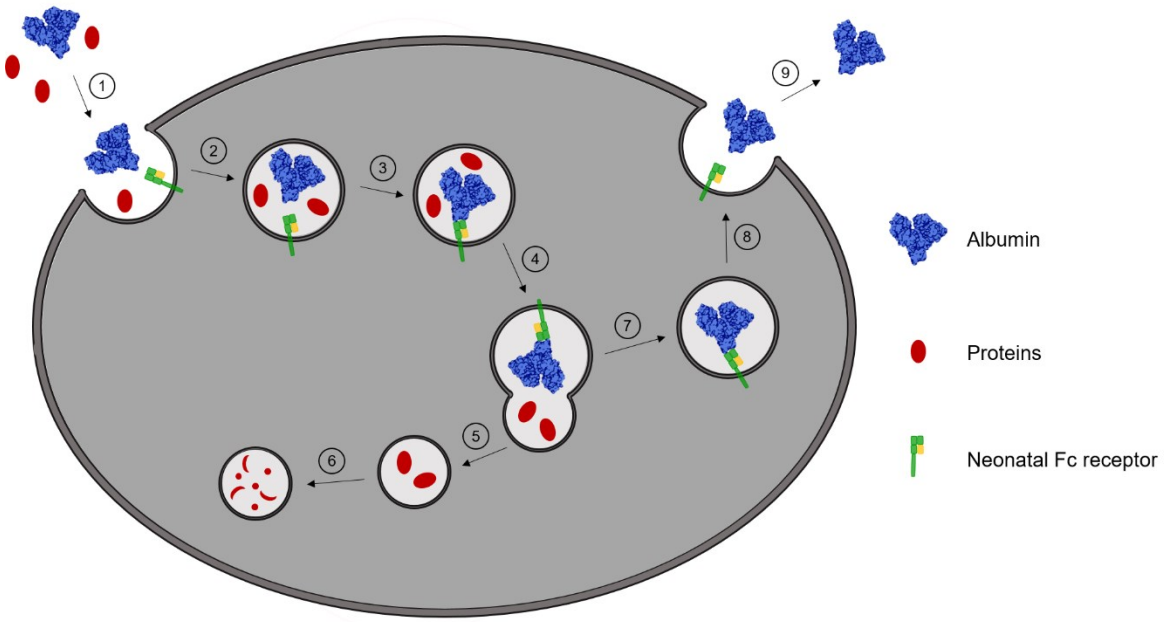

Figure 3: Schematic illustration of the recycling pathway of albumin via the neonatal Fc receptor (FcRn).
1) Albumin and other proteins are uptaken from the blood by the cell. 2) Endosomes are fomed. 3) Endosomes get acidified and albumin binds to the FcRn. 4) Endosomes are dividing in to two different ones. 5) Proteins that do not bind to the FcRn are 6) degraded. 7) Albumin bound to the FcRn is 8) transported to cell membrane and 9) released under physiological pH.

Hence, albumin drug conjugates can be used to extend the circulation time of the drug, reducing the application frequency of the drug e.g. to once-weekly formulations, which is already in use for diabetes therapy [197,411]. Nowadays, covalent attachment and non-covalent association of the drugs with albumin are being extensively studied [23,185,278]. Covalent conjugation is often associated with a modification of albumin itself, which can lead to a missing recognition by FcRn [338]. This is in contrast to non-covalent association whereby the drug administration is much more flexible. Unfortunately, the payload of drugs that can be associated with albumin is very low [151,414]. One possible solution for this problem would be to couple albumin to polymers that carry more than a single drug molecule to achieve higher payloads. This already has been achieved by covalent conjugation [281,322], without losing any of the affinity of albumin to the FcRn [5] and is therefore highly desirable for non-covalent conjugation. However, this approach is challenging since it can lead to distinct protein aggregation [161,423], which might result in faster hepatic clearance [279]. Nevertheless, drug conjugation to albumin can be a good strategy for transferring the pharmacokinetics of albumin to the respective conjugated drug.

1.5 Nucleic acids

The identification of nucleic acids can be dated back to 1869 [83]. After the discovery of the structure of DNA in 1953 [376] that the combination of (deoxy)ribose and phosphodiester is used for storing, copying and reading information in the field of biotechnology, biomedicine and nanotechnology [152,264,272,409]. DNA is highly stable [332] and the synthesis as well as the resulting sequence is accurately controlled by existing methods [41]. In the past few decades huge progress has been made with regards to nucleic acid-based therapeutics [171]. Gene therapy is one example for the usage of nucleic acids, whereby an unnatural or malfunctioning gene is compensated by a functioning version. Gene therapy is currently underway for patients suffering e.g. hemophilia B [92], cystic fibrosis [157], hand and neck squamous carcinoma [258] or Severe Combined Immunodeficiency Disease [54]. Besides gene therapy nucleic acids are used as antisense oligonucleotides to inhibit the expression of DNA and RNA. They can be unmodified nucleotides or chemically modified in respect to increase their stability, binding and specificity [368]. Further modifications lead to the development of nucleoside analogue oligonucleotides resistant to nuclease activity [221]. The general mode of action is to block the reading of the genetic information by specifically binding to the target DNA or RNA, which results in the inhibition of protein production critical to the disease without affecting non-involved proteins [399]. The first antisense oligonucleotide approved was fomivirsen to treat retinitis caused by human cytomegalovirus (HCMV) infection [252]. Viral replication is inhibited by the binding of the antisense oligonucleotide to the respective mRNA fragment involved in this process [141].

Another class of nucleic acid-based therapeutics are aptamers, which are single-stranded synthetic DNAs or RNAs consisting of 56-120 nucleotides but the specific target region is only 10-15 nucleotides long [81,128]. DNA aptamers are able to target nucleotides that code for various proteins [248], whereas RNA aptamers can directly bind to intracellular and extracellular small molecular ligands or proteins [124,189]. The antiviral effect of the aptamers already has been shown for different viruses such as HIV-1 [260,314,417], HBV [109,415], HCV [200,201], Influenza virus [130,235], HSV-1 and HSV-2 [75,129,239], as well as Ebola virus [29], dengue virus [58] and SARS [2,313].

RNA interference, short interfering RNAs (siRNA) and microRNAs (miRNA) are further tools of nucleic acid-based therapeutics. RNA interference works on the principal of post-transcriptional gene silencing to control the development as well as cellular processes [89]. Small RNAs of 20-30 nucleotides [53] can be divided into miRNAs, which regulate endogenous genes [233], and siRNA , which protect the genome from foreign nucleic acids such as transgenes, transposons as well as viruses [202]. Genes that should be silenced are specifically recognized by the small RNA leading to Watson-Crick base pairing. This makes it easy to program the silencing [273]. The most prominent representative of an antiviral miRNA is miravirsen, an anti-oligonucleotide of the HCV miRNA-122. Chronic HCV patients treated with miravirsen showed a dose-dependent and prolonged reduction of HCV RNA [254,276,413]. In case of siRNA ALN-RSV01 seems to be the most efficient antiviral agent against RSV, which targets the viral replication leading to a prevention of new or progressive bronchiolitis obliterans syndrome [131,273]. Major drawbacks of the

aforementioned nucleic acid-based therapeutics are the undefined pharmacokinetics and missing long-term effects, way of delivery, immunogenicity as well as toxicity.

For the treatment of HSV infections nucleoside analogues such as idoxuridine or acyclovir are used. Those are drugs with already known pharmacokinetics and safety profiles in humans. Until now, there are no studies on nucleic acids from those nucleoside analogues used for the treatment of HSV infections, representing another possible tool for antiviral treatment.

1.6 Scope of the study

Macromolecular prodrugs are designed to optimize the delivery of the active drug component to the sites of infection. Typically, they are composed of several copies of one or different drugs which are sometimes conjugated to a carrier component. Compared to other fields, studies investigating the value of macromolecular (pro)drugs are less common in antiviral research and therefore the application as antiviral treatments lags behind.

Macromolecular drugs, such as anionic polymers, have already been described to act as broad-spectrum antivirals. Until now, no study is available that deals with the structure-activity-relationship of such broadly active macromolecular drugs. Therefore, one aim of my study was to evaluate the structure-activity-relationship between anionic polymers and different enveloped viruses to get insights into the antiviral mechanism, the further design and development of new broad-spectrum antivirals.

Another goal of my study was to evaluate the (non)-covalent conjugation of the nucleoside analogue acyclovir, recommended for the treatment of herpes simplex virus infections, to albumin by the usage of a polymer as drug carrier to create high deliverable payload. The conjugation to albumin is used to possibly transfer its pharmacokinetics, such as extended body clearance time, to the drug. This might be an approach for the development of systemic applications for the treatment of herpes simplex virus infections, which can be helpful for severe progress of disease or immunocompromised patients.

Furthermore, an additional aim of my study was to test nucleic acid scaffolds, namely macromolecular prodrugs composed of nucleoside analogues already approved for the treatment of herpes simplex virus infections, and to assess their activity, stability and uptake.

2 Materials and Methods

2.1 Materials

2.1.1 Eukaryotic cells

Cell line	Description	Kindly provided by
A549 (ATCC® CCL-185)	Human epithelial-like lung cancer cell line. Cell line was used for the infection studies with clinical isolates of herpes simplex virus type 1 and type 2 and was obtained from the American Type Culture Collection (ATCC) [324].	
ELVIS™ (ATCC® CRL-12072)	Enzyme-Linked Virus-Inducible System. Baby Hamster Kidney cell line encoding lacZ gene, which is expressed upon infection via viral transactivator ICP10 [269].	
HEK293T (ATCC® CRL-3216)	Adherent human embryonic kidney cell line, transformed by adenovirus type 5 expressing simian virus 40 (SV40) large T-antigen [144]. This cell line was used to produce HIV-1 and pseudotyped retroviral particles.	
HeLa (ATCC® CCL-2)	The HeLa cell line is an adherent immortal cell line derived from cervical cancer cells from Henrietta Lacks. Horizontal gene transfer from human papillomavirus 18 (HPV18) to human cervical cells created the HeLa genome [217].	
HFF	Fibroblasts-derived from normal human foreskin.	Sina Lippold, group of Jens von Einem, Institute of Virology, Ulm University Medical Center, Ulm, Germany.
Huh7	Human hepatoma cell line [245,416]. Adherent cell line that is highly permissive for hepatitis C virus replication.	Stefan Pöhlmann, Heidelberg, Germany.

TZM-bl (ARRRP, 8129)	Human CD4+, CCR5+, and CXCR4+ HeLa-derived HIV-reporter cell line encoding the firefly luciferase and lacZ genes under control of the HIV-1 LTR promotor [377].	
Vero E6	Subclone of epithelial Vero cell line derived from African green monkey kidney that exhibits some degree of contact inhibition, and is thus useful in propagating slowly replicating viruses.	Jonas Schmidt-Chanasit, Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany.

2.1.2 Bacteria

Bacteria	**Description**
Escherichia coli XL-2 blue™	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F' *proAB* lacI^{q}ZΔM15 *Tn*10 (Tet^{r}) Amy Cam^{r}] (Agilent Technologies)
Escherichia coli XL-2 blue MRF'	Δ*(mcrA)183* Δ*(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* [F′ *proAB* lacI^{q}ZΔM15 *Tn*10 (Tet^{r})] (Agilent Technologies)

2.1.3 Viruses

Viruses	**Description**	**Kindly provided by**
HAdV5	Human adenovirus type 5 comprising a reporter cassette for enhanced green fluorescent protein (eGFP) [301].	Florian Kreppel, Institute of Gene Therapy, Ulm University Medical Center.
HIV-1	HIV-1 virus stocks were produced as described in Schandock, F. et al. (2017) [300].	
EBOV pp	Luciferase encoding retroviral particles harboring the respective glycoproteins of the viruses. Pseudotyped particles were produced as described in Schandock, F. et al. (2017) [300].	
Flu pp		
Lassa pp		
Lyssa pp		
Marburg pp		
SARS-CoV pp		

HSV-2 e(GFP) 333	Green-fluorescent HSV-2 strain 333. Between the convergent transcribed UL3 and UL4 genes an eGFP expression cassette is inserted. The generation was done by co-transfecting Vero E6 cells with genomic DNA from wildtype HSV-2 (333) and plasmid pUL3UL4-CMVeGFP83 (Taylor et al., 2007). Virus stocks were produced as described in Frich, C. K. et al. (2018) [114].	Patricia G. Spear, Department of Microbiology-Immunology, Northwestern University, Chicago, USA.
HSV-1	Isolated from bronchoalveolar lavage. Carries an unknown mutation in the viral polymerase (R1089Q).	Marlies Just group of D. Michel from Ulm University Medical Center Institute of Virology, Department of Virological Diagnostic, Ulm, GER. Resistance analysis was done by A. Schubert from from Ulm University Medical Center Institute of Virology, Department of Virological Diagnostic, Ulm, GER.
	Isolated from throat flush. Carries an acyclovir-resistance mutation (L297S) [119,195,304] and an unknown mutation (Y172 stop) both in the viral thymidine kinase.	
HSV-2	Isolated from vaginal swab. Wildtype virus.	
	Isolated from anus swab. Carries an acyclovir-resistance mutation (463C^5-1) [162].	
ZIKV MR766	Zika virus strain that was isolated in 1947 from a sentinel rhesus macaque [320].	J. Schmidt-Chanasit, Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany.
ZIKV FB-GWUH-2016	Zika virus strain that was isolated in 2016 from a human fetal brain with severe abnormalities [91].	O. Vapalahti (Department of Virology, University of Helsinki, Helsinki, Finland).
ZIKV PRVABC-59	Zika virus strain isolated from a Puerto Rican patient [193].	Obtained from Center of Centers of Disease Control and Prevention (CDC).

2.1.4 Plasmid DNA

All used plasmids contain an ampicillin or kanamycin resistance gene for selection in bacteria.

Plasmid	Description	Kindly provided by
pBRNL4-3 92 TH014.12	Plasmid encoding the HIV-1 NL4 3 provirus in which the V3-loop region was replaced by the V3-loop of the CCR5-tropic 92th014.12 isolate [255].	
pNL1_luc (NL4-3, delta env, fluc)	Plasmid encoding the HIV-1 NL4-3 provirus lacking a large part of env gene. Firefly luciferase reporter gene is cloned into nef [74]. Resulting viral particles lack envelope glycoproteins, are non-infectious, and express firefly luciferase in transduced cells.	
pLTR-PVenv (rabies virus PV strain env)	Expression vector expressing glycoprotein of Rabies Virus PV strain [108].	
pLTR-EBL1env (European bat lyssavirus env)	Expression vector expressing glycoprotein of EBL [108].	
Ebola GP (Ebola Virus glycoprotein)	Expression vector expressing EBOV glycoprotein [160].	Christine Goffinett, Twincore, Hannover.
Lassa GP	Expression vector expressing glycoprotein of Lassa [379].	
MARV pCAGGS-GP (Marburg Vector Surface Protein)	Expression vector expressing glycoprotein of MARV [160]	Stephan Becker, Institute of Virology, Marburg University.
SARS-CoV pCAG-GP	Expression vector expressing glycoprotein of SARS-CoV [133].	Stefan Pöhlmann, German Primate Center, University of Göttingen.
288+ HA (influenza env1)	Expression vector expressing the hemagglutinin of influenza virus [210]	
288+ HA (influenza env2)	Expression vector expressing the neuraminidase of influenza virus [210]	

2.1.5 Proteins and peptides

Antibody/Dye/Peptide/Protein	**Supplier**
CellMask™ Orange Plasma membrane Stain	Thermo Fisher Scientific
Goat anti-mouse IgG (H+L) Alexa Fluor® conjugate	Thermo Fisher Scientific
Goat anti-mouse IgG (H+L) HRP conjugate	Thermo Fisher Scientific
Mouse anti-flavivirus grouo antigen/protein E antibody (4G2)	Kindly provided by J. Schmidt-Chanasit, Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany.
Mouse HSV-2 gD Antibody (910)	Santa Cruz Biotechnology
Mouse HSV-1/2 gE Envelope Protein Antibody	Santa Cruz Biotechnology
Phalloidin-Atto 647N	ATTO-TEC

2.1.5.1 Enzymes

Ezymes	**Supplier**
Trypsin-EDTA 0.05% (1x)	PAN-Biotech
Trypsin-EDTA 0.25%, phenol red	Thermo Fisher Scientific
Trypsin-EDTA 0.5% (10x)	Thermo Fisher Scientific

2.1.6 Reagents and kits

2.1.6.1 Reagents

Reagent	**Supplier**
1 kilo base pair Plus DNA Ladder	Thermo Fisher Scientific
Acyclovir	Ratiopharm
Agar	Thermo Fisher Scientific
Agarose	Thermo Fisher Scientific
Ampicillin	Ratiopharm
Bacto tryptone	Becton Dickinson
Bacto yeast eytract	Becton Dickinson
BSA 10% Diluent/Blocking solution	KPL
Dimethylsulfoxid	Merck KGaA
Distilled H_2O (HPLC grade)	VWR
Dulbecco's Modified Eagle Medium (DMEM)	Thermo Fisher Scientific
Ethanol (99.8% purity)	VWR
Ethidium bromide	Applichem
FACS clean solution	BD Biosciences
FACS flow solution	BD Biosciences
FACS shutdown solution	BD Biosciences

Fetal bovine/calf serum (FCS)	Thermo Fisher Scientific
Glucose	Merck KGaA
Hoechst 33342 solution	Thermo Fisher Scientific
Hypochlorite (1%)	PanReac Applichem
Idoxuridine	European Pharmacopoeia
Incidine foam	ECOLAB Healthcare
Isopropanol	VWR
Kanamycin	Thermo Fisher Scientific
L-Glutamine	Thermo Fisher Scientific/PAN-Biotech
Lipofectamine® 3000	Thermo Fisher Scientific
Methanol	Sigma-Aldrich
Methylthiazolyldiphenyl-tetrazolium bromide (MTT), powder, 5 mg/ml in PBS	Sigma-Aldrich
Non-essential amino acids	Sigma-Aldrich
Opti-MEM™ I Reduced Serum Medium	Thermo Fisher Scientific
Paraformaldehyde	Merck KGaA
Penicillin-Strptomycin	Thermo Fischer Scientific/PAN-Biotech
Phosphate buffered saline (PBS), no calcium, no magnesium	Thermo Fischer Scientific
Potassium chloride (KCl)	PanReac AppliChem
Roti®-Load DNA	Carl Roth
Sodium chloride (NaCl)	Sigma-Aldrich
Sodium pyruvate	Thermo Fisher Scientific
Sulfuric acid (H_2SO_4)	Merck KGaA
SureBlue™ TMB 1-Component Microwell Peroxidase Substrate	KPL
TAE buffer	5Prime
Trifluridine	British Pharmacopoeia
Triton X-100	Sigma-Aldrich
Tween®	Sigma-Aldrich
X-VIVO 15 chemically defined, serum-free medium	Biozym

2.1.6.2 Kits

Kit	**Supplier**
Calphos mammalian transfection kit	Clontech
CellTiter-Glo® Luminescent Cell Viability Assay	Promega
Gal-Screen™ β-Galactosidase Reporter Gene Assay System for Mammalian Cells	Thermo Fisher Scientific
Lipofectamine® 2000 Reagent	Thermo Fisher Scientific
Luciferase Assay System	Promega

PCR Mycoplasma Test Kit	Applichem/BioFroxx
Restriction endonucleases and respective buffers	New England Biolabs
Wizard® Plus Midipreps DNA Purification System	Promega

2.1.7 Solution and buffers

Cell-based ZIKV immunodetection assay

Antibody buffer	10% (v/v) FCS and 0.3% (v/v) Tween 20 in PBS
Washing buffer	0.3% (v/v) Tween 20 in PBS

ZIKV/HSV-1/-2 immunostaining

Antibody buffer	1% (v/v) BSA in PBS
Blocking buffer	5% (v/v) FCS and 1% (v/v) BSA in PBS
Permeabilization solution	0.1% (v/v) Triton X-100 in PBS

Agarose gel electrophoresis

50x TAE buffer	2 M Tris-HCl, 1 M acetic acid and 0.1 M EDTA (final pH 8.3)
Agarose gel loading buffer	0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 30% (v/v) glycerol in TAE buffer

2.1.8 Media

Bacteria culture media

LB agar	1.5% (w/v) agar with 0.01% (w/v) ampicillin or kanamycin in LB medium
LB medium	1% (w/v) bacto tryptone, 0.5% (w/v) bacto yeast extract, 0.8% (w/v) NaCl, 0.1% (w/v) glucose, and 0.01% (w/v) ampicillin or kanamycin added prior to use
S.O.C. medium	Thermo Fisher Scientific

Cell culture media

DMEM culture medium	Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin.

Huh7 culture medium	DMEM supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2 mM L-glutamine, 1x non-essential amino acid, 100 units/ml penicillin, and 100 µg/ml streptomycin.
Vero E6 culture medium	DMEM supplemented with 2.5% (v/v) heat-inactivated fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 1x non-essential amino acid, 100 units/ml penicillin and 100 µg/ml streptomycin.
Vero E6 serum-free medium	X-VIVO™ supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 1x non-essential amino acid, 100 units/ml penicillin and 100 µg/ml streptomycin.
A549/HFF serum-free medium	X-VIVO™ 15 chemically defined medium supplemented with 2 mM L-glutamine as wells as 100 units/ml penicillin and 100 µg/ml streptomycin.

2.1.9 Consumables

Consumable	**Supplier**
µ-Slides 8 well	Ibidi
Cell culture flasks (T25, T75, T175)	Sarstedt
Cell culture 6-well plates (F)	Sarstedt
Cell culture 96-well plates (F, R, V)	Sarstedt
Cryo tubes	Sarstedt
Costar® 96-well cell culture plates, black with clear flat bottom, TC-treated	Corning
FACS tubes	BD Biosciences
Gloves	VWR
Pipette tips (10 µl, 1000 µl)	Nerbe plus
Pipette tips (10 µl, 20 µl, 200 µl)	Eppendorf
Reaction tubes (1.5 ml, 2 ml)	Eppendorf
Reagent reservoirs	VWR
Serological pipettes (2 ml, 5 ml, 10 ml, 25 ml, 50 ml)	Sarstedt
Tubes (15 ml, 50 ml)	Sarstedt
LumiNunc™ 96 well polystyrene plates	Thermo Fisher Scientific
Wipes	Kimberly-Clark

2.1.10 Technical equipment

Device	**Manufacturer**
BD FACSCanto™ II Cell Analyzer	BD Biosciences
BINDER BD 53 Incubator	Binder

BINDER BD720 Incubator	Binder
Centrifuge 5417R Rotor: F 45 30 11	Eppendorf
Centrifuge 5804R Rotor: 96 well plate A-2-MTP	Eppendorf
Centrifuge 5810R Rotor: A 4 81	Eppendorf
Cytation™ 3 Cell Imaging Multi-Mode Reader	BioTek
Discovery comfort 12-channel pipettes	HTL Lab Solutions
Discovery comfort pipettes	HTL Lab Solutions
DM IL LED microscope	Leica microsystems
FiveEasy™ pH meter	Mettler Toledo
Forma Steri-Cult™ CO_2 incubator	Thermo Fisher Scientific
Gel Doc™ XR System	Bio-Rad
Hemacytometer cover glasses	VWR
Hemacytometer Neubauer improved	VWR
Heracell™ 240 CO_2 incubator	Thermo Fisher Scientific
Herasafe™ KSP laminar flow hood	Thermo Fisher Scientific
HXP 120 C	Pulch + Lorenz
KERN ABJ 120 4M scale	Kern & Sohn
LASOS® RMC Ar-Ion Laser	LASOS
LSM 710 confocal laser scanning microscope	Zeiss
Microscope camera MC120HD	Leica Microsystems
NanoDrop™ 2000 UV-Vis Spectrophotometer	Thermo Fisher Scientific
Orion II Microplate Luminometer	Titertek Berthold
Pipettus-Aku	Hirschmann Laborgeräte
PowerPac™ Basic Power Supply	Bio-Rad
PowerPac™ HC High-Current Power Supply	Bio-Rad
Rotilabo® mini-centrifuge	Carl Roth
Sartorius® CERTOMAT® IS	Thermo Fisher Scientific
Scout® Pro SPU402 Scale	Ohaus
Sub-Cell® GT Cell	Bio-Rad
Thermomixer comfort	Eppendorf
Transferpette® S -12 - Solutions	Brand
UV transilluminator, 366nm	Syngene
VMax Kinetic ELISA Microplate Reader	Molecular Devices
Vortex mixer	VWR
Vortex-Genie 2®	Scientific instruments

2.1.11 Software

Software	Supplier
Adobe® Illustrator	Adobe
BD FACSDiva™	BD Biosciences
Gen5	BioTek
GraphPad Prism 5	GraphPad Software
Huygens Professional	
LSM 710 Release version 5.5SP1	Zeiss
Microsoft Office	Microsoft
Simplicity 4.02	Titertek Berthold
Softmax Pro™	Molecular Devices
ZEN 2.3 (blue edition) 2016	Zeiss
ZEN software 2010	Zeiss

2.2 Methods

2.2.1 DNA

2.2.1.1 Plasmid DNA generation

Plasmid DNA was prepared by alkaline lysis of grown bacterial cultures. Therefore, Wizard® Plus Midiprep Kit was used as recommended by the manufacturer. Purity and concentration of plasmid DNA was determined using the NanoDrop™ 2000 Spectrophotometer.

2.2.1.2 Restriction digestion

Isolated plasmid DNA was analyzed for correct size by restriction digest. Appropriate endonucleases and buffers according to the New England BioLabs' instructions followed by subsequent gel electrophoresis were used.

2.2.1.3 Agarose gel electrophoresis

Plasmid DNA and fragments were separated on a 0.7% (w/v) agarose gel mixed with ethidium bromide. 20 µl digested samples were ran in parallel to 5 µl DNA ladder with 20% (v/v) loading dye on the gel in TAE buffer for 30 minutes at 120 V (Voltage PowerPAC Basic Power Supply) in gel electrophoresis cell (Sub-Cell® GT Cell). DNA bands were visualized with the Gel Doc XR.

2.2.2 Bacteria

2.2.2.1 Transformation of bacteria

For the amplification of expression and proviral vectors, bacteria were transformed with plasmid DNA. 0.5 µl of plasmid DNA preparations were incubated with 5 µl Escherichia

coli XL-2 blue™ or XL-2 blue MRF' for 20 minutes on ice. This is followed by a 30 second heat-shock at 42°C and another 2.5 minutes of cooling on ice. 200 µl of S.O.C. medium was added to the transformed bacteria and they were incubated for 40 minutes at 37°C (XL-2 blue) or 30°C (XL-2 blue MRF') under 400 rpm of shaking in a Thermomixer to stimulate bacterial growth. After incubation, 70 µl of bacteria was plated on LB agar plates containing appropriate antibiotics (ampicillin or kanamycin) for selection and plates were incubated overnight in an incubator at 37°C (XL-2 blue) or 30°C (XL-2 blue MRF').

2.2.2.2 Culture of bacteria

To amplify transformed bacteria for midi preparations, single colonies of bacteria were cultured in 150 ml LB medium supplemented with 100 mg/L antibiotic (ampicillin or kanamycin, respectively) and incubated on a shaker for 12-16 hours at 37 °C (XL-2 blue) or for 24 hours at 30 °C (XL-2 blue MRF').

2.2.3 Eukaryotic cells

2.2.3.1 Cell culture of adherent cells

Cells were cultured in T75 or T175 cell culture flasks at 37 °C in a 5% CO_2 humidified incubator. Vero E6 cells were cultured in Vero E6 culture medium, A549, ELVIS™, HEK293T, HeLa, HFF and TZM-bl cells in DMEM culture medium and Huh7 cell in Huh7 culture medium. Cells were passaged at a confluence of 90%. To this end, cells were detached by trypsin, resuspended in the respective medium and split 1:5 or 1:10. A549 cells were not kept in culture longer than 25 passages, primary HFF cells were not kept in culture longer than 30 passages.

2.2.3.2 Cell stocks thawing and freezing

Cell lines were slowly thawed from -80°C and poured into 10 ml of the respective culture medium. To remove remnants of freezing medium prior culturing, cells were centrifuged for 3 min at 340 x g and the cell pellet was resuspended in 10 ml culture medium and transferred to a T25 flask. Cell stocks were prepared by resuspending 1-5x10^6 cells per ml freezing medium (FCS containing 10% DMSO) carefully and frozen down in 1 ml aliquots quickly at -80°C in CryoPure tubes.

2.2.3.3 Mycoplasma test

To exclude mycoplasma contaminations, a PCR was performed according to the manufacturer's instructions of the PCR Mycoplasma Test Kit. If contaminated, cells were trashed and uncontaminated stocks thawed.

2.2.3.4 CellTiter-Glo® Luminescent cell viability assay

The CellTiter-Glo® assay system determines cell viability based on cellular ATP levels. The assay was performed in 96 well plates. Therefore, the plates were exposed to room

temperature. After 15 min 50 µl of buffer-substrate-mix of CellTiter-Glo® luminescent cell viability assay was added to each well containing 100 µl medium. After 10 min incubation in the dark, the solution was transferred into a white LumiNunc™ 96 well plate and ATP-dependent chemiluminescence was measured using Orion II microplate luminometer (Software Simplicity, 0.1 s measurement).

2.2.3.5 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) cell viability assay

The 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay determines the metabolic activity of cells. Live cells reduce the yellow MTT salt by NAD(P)H-dependent oxidoreductase system causing the formation of insoluble purple formazan crystals. The assay was performed in 96 well plates. Therefore, the medium was discarded and 100 µl of 0.5 mg/ml MTT-PBS solution was added to the cells. After 2 h, the cell-free supernatant was discarded and formazan crystals were dissolved in 100 µl DMSO:Ethanol (1:2). Absorption was detected at 490 nm and corrected by the background absorption at 650 nm using Vmax kinetic microplate reader.

2.2.4 Synthesis of macromolecular prodrugs and analyzing methods

2.2.4.1 Synthesis of macromolecular antiviral agents - polyanions

The polymers were synthesized as published in Schandock, F. et al. [300]. Briefly, polymers with negatively charged side chains are known to act as viral inhibitors. This led to a study where the largest anionic polymer library, to the best of own knowledge, ever been reported, was synthesized from commercially available as well as custom made monomers with anion functionalities. The polymer synthesis was performed using the versatile and robust technique reversible addition-fragmentation chain transfer polymerization (RAFT). The anionic functionalities belong to the following three groups: carboxylate, phosphate/phosphonate and sulfonate. Variations in the chosen monomers furthermore included differences in the overall hydrophobicity of the monomer [300,386]. The polymers were synthesized by Camilla Kaas Frich and Kaja Borup Løvschall, Department of Chemistry, Aarhus University, Denmark.

2.2.4.2 Synthesis of antiviral macromolecular prodrugs of acyclovir and release studies

The macromolecular prodrugs of acyclovir were synthesized as published in Frich, C. K. et al. [114]. In brief, Acyclovir (ACV) was converted into a redox responsive macromolecular prodrug by copolymerizing a methacrylate based monomer of ACV with 2-hydroxypropyl methacrylate (HPMA) by reversible addition-fragmentation chain transfer polymerization (RAFT polymerization). The ACV monomer was synthesized containing a disulfide linkage that is stable extracellularly but upon entering the cell, intracellular glutathione reduces the disulfide linkage initiating a cyclization cascade that releases pristine acyclovir [180,280,288]. Synthesis was performed by Raoul Walther, Department of Chemistry, Aarhus University, Denmark.

A dilution of PHPMA-ACV polymer with a concentration of 1 mg/ml in PBS (pH 7.4, 10 mM) was prepared. The solution was incubated with or without 5.0 mM glutathione at 37°C for 3 h. Spin filtration was used to remove the polymer (MWCO=3 kDa) and analysis of the filtrate was performed with HPLC. Conversion of the AUC (254 nm) of acyclovir to molar concentration was done with a standard curve. The assay was performed by Raoul Walther, Department of Chemistry, Aarhus University, Denmark.

2.2.4.3 Synthesis of macromolecular prodrugs of nucleoside analogues and enzymatic digestion

Prodrugs of nucleoside analogues (therapeutic nucleic acids) were synthesized as published in Krüger, F. et al. [186]. Briefly, the synthesis was done by automated oligonucleotide synthesizer on solid support [41]. In general, to construct the certain length of a particular nucleic acid, it is important to synthesize corresponding phosphoramidite monomers. Therefore, two phosphoramidite monomers in two chemical synthetic steps from commercially available Idoxuridine and Trifluridine antiviral nucleosides were produced [126,308]. The automated oligonucleotide synthesis is a cascade of various chemical reactions. This provides the opportunity to feed the different type of phosphoramidite monomers in only one nucleic acid sequence. Therefore, also chimeric sequences of Thymidine with Idoxuridine and Trifluridine nucleosides were synthesized. The synthesis of prodrugs were performed on 200 nmol scale and one prodrug was synthesized various times in order to achieve the required quantity. After the synthesis, oligonucleotides were cleaved from the solid support and followed the standard or modified protocol for purification. Synthesis was performed by Vipin Kumar, iNano Interdisciplinary Nanoscience Centre, Aarhus University, Denmark.

The enzymatic digestion of therapeutic nucleid acids (TNA) was done with Phosphodiestearase I from Crotalus adamanteus venom (Sigma P3134). The enzyme was added to the different TNAs (I_{14} = 25 µM; F_{14} and T_{14} = 35 µM) in TrisHCl buffer to a final concentration of 35 µg/ml. To dissect background phosphatase activity of Phosphodiestearase I thymidine 5'P monophosphate (0.4 mM) was used as control. After 2 h incubation at 37°C the samples were spin-filtered (cut-off of 100 kDa) before analysis with HPLC or LC-MS was performed. The same was done for analyzing the nuclease activity of FCS. Therefore, TNAs (I_{14} = 25 µM; F_{14} and T_{14} = 35 µM) were incubated in MEM supplemented with or without 10% FCS for 2 h at 37°C. Spin-filtration was done with a 3 kDa cut-off filter and HPLC as well as LC-MS used for analysis. Assays was performed by Pere Monge Marcet from Department of Chemistry and iNano Interdisciplinary Nanoscience Centre, Aarhus University, Denmark.

2.2.5 Virology methods

2.2.5.1 Generation of HEK293T cells-derived virus stocks

Virus stocks were generated by transient transfection of HEK293T cells using the calcium phosphate precipitation technique [80]. Therefore, the calphos mammalian transfection kit

was used. Briefly, HEK293T cells were seeded in 6 well plates. The day after, medium was replaced with fresh medium when cells were at a confluence of 60-80%. 5 µg (for 2 ml medium per 6 well) of proviral plasmid DNA was diluted in 125 mM $CaCl_2$ and added dropwise to an equal volume of 2x HBS. After 5 minutes of incubation the DNA precipitate was added dropwise to the culture medium. 12-16 hours post transfection medium was replaced with fresh DMEM (supplemented with 2.5% (v/v) FCS) and incubated for another 24 hours. The supernatant, containing the produced virions, was centrifuged (3 min, 330 x g) to remove cellular debris and transferred into reaction tubes. Virus stocks were stored at -80 °C.

2.2.5.2 Generation of HEK293T cells-derived pseudotyped virus stocks

Lentiviral pseudotyped virus stocks were generated by transient transfection of HEK293T cells using the calcium phosphate precipitation technique as described above (2.2.5.1) [80]. For pseudotyping, cells were cotransfected with a crippled lentiviral vector encoding firefly luciferase and lacking a large part of env gene (pNLEnv-1) [218] and expression plasmids for different viral glycoproteins from either Influenza H5N1 [210], Lassa [379], Lyssa and Rabies [108], Ebola and Marburg [160], or SARS-CoV [133]. 12 hours post transfection medium was replaced with fresh DMEM supplemented with 2.5% (v/v) FCS and cells were incubated for further 48 h. Afterwards virus stocks harvested by centrifugation of the supernatant (3 min, 330 x g) to remove cellular debris and then transferred into reaction tubes for storing the virus stocks in -80°C.

2.2.5.3 ZIKV virus propagation

ZIKV was propagated by inoculation of 70% confluent Vero E6 cells in T175 cell culture flasks for 2 h in 5 ml medium. Afterwards, 35 ml fresh medium was added and the cells were cultured for 3 to 5 days by monitoring the cytopathic effect (CPE) by light microscopy. Virus was harvested after a CPE-dependent detachment of 70% of the cells. Supernatant was centrifuged for 3 min at 330 x g to remove cellular debris, aliquoted and stored at -80°C as virus stocks.

2.2.5.4 HSV virus propagation

HSV-2 eGFP was propagated by inoculation of 70% ELVIS^{TM} cells in T175 cell culture flasks, HSV-2 eGFP and clinical isolates of HSV-1 (from broncho-alveolar lavage and throat flush) and HSV-2 (from vaginal and anus swab) were propagated by inoculation of 70% Vero E6 cells. Therefore, the medium was replaced by 35 ml fresh medium supplemented with 200 µl of virus stock of HSV-2 eGFP or clinical isolates of HSV-1 and HSV-2. Cells were cultured for 2 to 4 days by monitoring the CPE by light microscopy. Virus was harvested after 70% of detached cells. Supernatant was centrifuged for 3 min at 330 x g to remove cellular debris, aliquoted and stored at -80°C as virus stocks. Tissue culture infectious dose 50 ($TCID_{50}$) of each stock was determined by infection of Vero E6 cells with serially diluted virus stocks and calculated according to Reed and Muench [277] as described in 2.2.5.5.

2.2.5.5 $TCID_{50}$ endpoint titration of HSV-1, HSV-2 and HSV-2 eGFP

For determination of the infectious HSV titer, 6,000 Vero E6 or ELVIS™ cells were seeded per well in 96 flat bottom well plates in 100 µl medium and incubated overnight. 80 µl fresh medium was added the next day. For endpoint tissue culture infectious dose 50 ($TCID_{50}$) determination HSV samples were titrated 10-fold, and 20 µl of each dilution was used for inoculation of Vero E6 cells. This endpoint titration resulted in final HSV dilutions of 10x to 10^9x on the cells in triplicates or sextuplicates. Cells were then incubated and the cell viability assay MTT described in 2.2.3.5 was used to determine the titer after 7 days. For determination of the infection rates, sample values were subtracted from untreated control and untreated control was set to 100%.

2.2.5.6 HIV-1 infectivity assay and effect of polyanions on HIV-1 infection

HIV-1 infectivity was measured by infection of TZM-bl reporter cells. This cell line is stably transfected with an LTR-lacZ cassette and expresses CD4, CXCR4, and CCR5. Upon infection with HIV-1, the viral protein Tat is expressed which activates the long terminal repeat (LTR) resulting in the expression of β-galactosidase. Virus stocks of CCR5-tropic HIV-1 NL4-3 92TH014.12 [255] were generated as described in 2.2.5.1. In "virus treatment" assays, HIV-1 particles were incubated with anionic polymers (0-50 µg/ml) for 10 min at 37°C. Afterwards, these mixtures were used for infection of 10,000 TZM-bl cells in triplicates seeded the day before into 96-well plates. In "cell treatment" assays, 0-50 µg/ml of the anionic polymers were incubated with the cell for 1 h at 37°C in triplicates prior infection. HIV-1 infection rates were determined three days post infection by detecting β-galactosidase activity in cellular lysates using the Gal-Screen™ β-Galactosidase Reporter Gene Assay System for Mammalian Cells. Therefore, the medium was discarded and 40 µl of diluted Gal-Screen™ substrate/bufferA (1:8 in PBS) was added. Cells were lysed during the 30 min of incubation at room temperature and the substrate converted by the released β-galactosidase. Subsequently to the incubation, 35 µl were transferred in a white 96 well plate. The substrate conversion was measured as relative light units per second (RLU/s) using the Orion II microplate luminometer. Reporter gene activities (recorded as relative light units per second) obtained from infected cells containing no polyanion were used as control (100%) and triplicates expressed as mean ± standard error derived from two independent experiments.

2.2.5.7 Pseudoparticle infectivity assay and effect of polyanions on pseudoparticles

Pseudoparticles were generated as described in 2.2.5.2. The crippled lentiviral vector encoded a firefly luciferase. Upon infection, luciferase is expressed and can be quantified by the luciferase assay system. Therefore, 10,000 HEK293T (for Influenza pseudoparticles), 10,000 Huh7 cells (for Lyssa, Rabies, Ebola and SARS pseudotyped lentiviruses) or 6,000 Vero E6 cells (for Lassa pseudoparticles) were seeded the day before infection in 96-well plates. The day after, cells were treated with 50 µg/ml of the polymers for 1 h at 37°C prior infection with the respective pseudoparticles. After 2-3 days post infection, medium was discarded and 40 µl of Luciferase Assay cell culture lysis reagent was added. The cells

therefore were lysed at room temperature for 10 min and firefly luciferase released. Afterwards, 30 µl were transferred into a white 96 well plate and 50 µl Luciferase Assay Substrate was added. Immediately afterwards, chemiluminescence was measured as relative light units per second (RLU/s) using the Orion II Microplate Luminometer. All values represent % infection rates relative to control infections containing no polyanion (100%). Reporter gene activities (relative light units per second; RLU/s) were derived from triplicate infections minus background activities derived from uninfected cells.

2.2.5.8 ZIKV infectivity assay and effect of polyanions on ZIKV

ZIKV induces a cytopathic effect which can be determined by the MTT-based colorimetric detection assay [244]. Initial testing of the effect of polyanions was performed by seeding 6,000 Vero E6 cells into 96 well plates. The day after, for the "cell treatment" 0-100 µg/mL of the polymers were added in triplicates to the cells and incubated for 90 min at 37°C prior infection ZIKV strain MR766, a ZIKV strain that was isolated in 1947 from a sentinel rhesus macaque [87]. For the "virus treatment" 0-100 µg/ml of the polymers were preincubated with the virus ZIKV MR766 for 15 min at 37°C before using these mixtures to infect the cells in triplicates. 3 days post infection MTT was performed as described in 2.2.3.5 to indirectly determine ZIKV induced cell death and to assess protection by polyanions.

2.2.5.9 Cell-based ZIKV immunodetection assay and effect of polyanions on ZIKV

The effect for the polymers was reevaluated with higher concentrations up to 200 µ/ml in a cell-based ZIKV immunodetection assay. "Cell" and "virus treatment" was performed as already described in 2.2.5.8. After 36 hours post infection, cells were washed with PBS and fixed with 4% paraformaldehyde (PFA) for 20 minutes at room temperature. Afterwards they were permeabilized with cold methanol for 5 minutes at 4 °C, and washed with PBS. Subsequently, cells were incubated with mouse anti-flavivirus antibodies 4G2 in antibody buffer for 1 hour at 37 °C. Next, wells were washed 3 times with washing buffer and afterwards incubated with a HRP-coupled anti-mouse antibody (1:20,000) for one hour at 37°C. Cells were washed 4 times with PBS and TMB substrate was added. After 5 minutes of incubation at room temperature, reaction was stopped with 0.5 M sulfuric acid. Absorption was measured at 450 nm and baseline corrected at 650 nm using an ELISA microplate reader.

2.2.5.10 Flow cytometry of polyanions and ZIKV

To study the interaction of ZIKV with the FITC coupled PEAA flow cytometry was used. Different ZIKV MR766 amounts were incubated with 200 µg/ml of PEAA-FITC in FACS tubes for 15 min at room temperature. There formed polyanion-ZIKV cluster were analyzed by using FACS.

2.2.5.11 Effect of polyanions on human adenovirus infection

The effect of polyanions on E1-deleted replication deficient human adenovirus type 5-based vector containing a HCMV promotor-controlled eGFP expression cassette was assessed by using flow cytometry as described in Schandock, F. et al., 2017 [300]. The experiments were performed by Florian Kreppel at the Ulm University, Germany.

2.2.5.12 HSV-2 eGFP infectivity assays and effect of macromolecular prodrugs

The effect of macromolecular prodrugs (MPs) (polyanions and MPs of acyclovir) was assessed by generation of HSV-2 eGFP strains by infecting ELVIS™ cells as described in 2.2.5.4. The DNA of ELVIS™ cells carry a promoter gene that is chimerically linked to an *Escherichia coli* LacZ reporter gene. HSV leads to an induction of the promoter and the production of the LacZ product β-galactosidase, which can be used to determine the infection rates after 36-48 h post infection by using the Gal-Screen™ β-Galactosidase Reporter Gene Assay System for Mammalian Cells. Briefly, supernatant was discarded and 40 µl of diluted Gal-Screen™ substrate/bufferA (1:4 in PBS) was added. Cells were lysed for 30 min at room temperature and the substrate converted by the released β-galactosidase. After incubation, 35 µl were transferred in a white 96 well plate. The substrate conversion was measured as relative light units per second (RLU/s) using the Orion II microplate luminometer. Reporter gene activities (recorded as relative light units per second) obtained from infected cells containing no polyanion or MPs of acyclovir were used as control (100%).

For the effect of polyanions two approaches were performed. In the "virus treatment" assays, HSV-2 eGFP were incubated with anionic polymers (0-50 µg/ml) for 10 min at 37°C. Afterwards, these mixtures were used for infection of 6,000 ELVIS™ cells in triplicates seeded the day before into 96-well plates. In "cell treatment" assays, 0-50 µg/ml of the anionic polymers were incubated with the cell for 1 h at 37°C in triplicates prior infection. Triplicates are expressed as mean ± standard error derived from two independent experiments.

For the effect of MPs of acyclovir the "cell treatment" assay was performed. ELVIS™ cells were incubated with MPs with ranging concentrations from 0-100 µg/ml and for acyclovir as control ranging from 0-1000 µM for 1 h at 37°C in triplicates prior infection with HSV-2 eGFP. Triplicates are expressed as mean ± standard error derived from three independent experiments.

2.2.5.13 In vivo biodistribution of macromolecular prodrugs of acyclovir

12 ffemale, 8 weeks old BALB/cJRj mice (janvier-labs.com) were used to study the biodistribution and blood residence time of macromolecular prodrugs of acyclovir after subcutaneous injection in the scruff of the neck or intravenous injection in the tail. Injected have been DSPE-PHPMA-Cy7 (2 mg/kg), PHPMA-Cy7 (0.4 mg/kg), PHPMA-Cy7-albumin (4 mg/kg) or PBS. Until 7 days post injection blood samples were taken from the tail tip or retroorbital plexus from 4 mice of each group. After separation of plasma and cells by centrifugation the amount of compound in the plasma was determined by fluorescence signal. Fluorescence of PBS was subtracted from all other samples and compound portion in the plasma was related to fluorescence intensity in the plasma. The compound amount is

related to its maximum fluorescence at the respective injection route and expressed as the mean radiance (photons/s/cm2/sr). Furthermore, after 1 h, 24 h and 7 days post injection 4 mice were euthanized and blood was eliminated from organs with PBS perfusion through the heart. Image acquisition of the single organs was performed with *in vivo* imaging system (IVIS, PerkinElmer, MA, USA) with a 745/820 nm Cy7-filterset and images were analyzed with Living Image software v4.3 (PerkinElmer). Assays were performed by Anna Folkmar Halling Andersen, Department of Infectious Diseases/Department of Clinical Medicine, Aarhus University Hospital and Department of Clinical Medicine, Aarhus University.

2.2.5.14 Effect of macromolecular prodrugs of acyclovir on genital HSV-2 infection in mice and virus plaque assay

To prevent pregnancy the female mice were treated with 2 mg Depo-Provera (Pfizer) 5 days prior intravaginal infection with HSV-2 (20 µl of $6.7x10^4$ plaque forming units) under anaesthesia with Isoflurane (Abbott) for 10 min. After 5 h mice were treated with DSPE-PHPMA-ACV, PHPMA-ACV or acyclovir adjusted to a molar amount of 60 mg acyclovir/kg mouse. DSPE-PHPMA-ACV and PHMPA-ACV were mixed with equimolar amounts of murine serum albumin prior injection. 48 h post infection vaginal washed of uninfected, untreated and infected, treated mice were collected by rinsing the vagina twice with 40 µl IMDM (end volume 250 µl). Until analysis samples were kept on -70°C. Euthanasia of mice was done by cervical dislocation 7 days post infection. To determine the severity of infection mice were observed and examined every day. The following scale was used for determination [223]: 0: healthy; 1: genital erythema; 2: moderate genital inflammation; 3: purulent genital lesion/or generally bad condition; 4: hind limb paralysis or generally very poor condition. Mice with a score higher than 3 were euthanized by cervical dislocation.

For determination of viral titer the medulla spinalis were removed and homogenized before analyzing the viral titers. Titers were determined on 1.2×10^6 Vero cells per well seeded in MEM supplemented with 5% FCS. On day 2 cells homogenized medulla spinalis or vaginal washed were serially diluted prior infection of cells (100 µl). After 1 h incubation, MEM was supplemented with 0.2% human immunoglobulin (ZLB Behring GmBH) and 5 ml added to the cells. 2-3 days post infection cells were stained with 0.03% methylene blue and plaques were counted. The results were stated as plaque forming units per vaginal wash or per g tissue medulla spinalis. Assays were performed by the group of Søren R. Paludan, Department of Biomedicine, Aarhus University.

2.2.5.15 HSV infectivity assays and effect of Therapeutic Nucleic Acids

HSV is a cytopathic virus. Among infection the cells start to form syncytia, polykaryocyte cells or start to die [246,383]. This approach can be used to determine antiviral effects with cell viability assays [242,244]. The effect of therapeutic nucleic acids was therefore determined with two different cell viability assays: CellTiter-Glo® (CTG) luminescent cell viability assay and the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. 6,000 Vero E6 cells per well were seeded in 96-well flat bottom plate in 100

µl medium. The next day, medium was removed and 70 µl fresh medium was added. Therapeutic nucleic acids (TNA) were diluted from 0-20 µg/ml and added to cells in triplicates for 2 h at 37°C prior infection with HSV-2 eGFP or clinical isolates of HSV-1 and HSV-2. 72 h later either the ATP levels (with CTG assay) or the metabolic activity (with MTT assay) were determined as described in 2.2.3.4 for CTG assay and in 2.2.3.5 for MTT assay. Infection rates were determined by subtracting sample values from uninfected control and untreated control was set to 100%. Triplicates are expressed as mean ± standard error derived from three independent experiments.

For studying extracellular stability of the TNA, 6,000 Vero E6 cells per well were seeded in 96-well flat bottom plate in 100 µl medium. The day after, medium was discarded, cells were carefully washed with PBS and 70 µl chemical defined medium without FCS (X-VIVO^{TM}) was added. TNAs were diluted from 0-20 µg/ml and added to cells in triplicates for 2 h at 37°C prior infection with clinical isolates of HSV-2. Metabolic activity was determined 72 h post infection as indicated above with the MTT assay. Infection rates were determined by subtracting sample values from untreated control and untreated control was set to 100%. Triplicates are expressed as mean ± standard error derived from two independent experiments.

2.2.6 Imaging Methods

2.2.6.1.1 Immunostaining and confocal microscopy of ZIKV infected cells

To visualize ZIKV infection, laser scanning microscopy was performed. Therefore, 24,000 Vero E6 cells were seeded into an 8-well Ibidi slide. One day later, for the "cell treatment" cells were incubated with PSVBS or PVBzA. Infection was accomplished after 90 min incubation at 37°C with ZIKV MR766. In the set-up of "virus treatment", ZIKV MR766 was incubated with PEAA or fluorescent PEAA Rhodamine B copolymer (PEAA-Rho B) for 15 min. The mixture was used to infect Vero E6 cells. 3 days post infection in both experimental set-ups the cells were fixed for 10 min at 4°C with 4% paraformaldehyde (PFA). Permeabilization was performed with 0.1% Triton-X-100. Afterwards, blocking buffer was used to incubate the cells for 30 min at room temperature. ZIKV infection was visualized with mouse anti-flavivirus antibodies 4G2 (1:100 (v/v)) and secondary Alexa Flour 488® goat anti-mouse IgG (1:1,000 (v/v)). Both antibodies were diluted in antibody buffer for immunostaining and incubated for 45 min at room temperature, respectively. Next, nucleus was stained with Hoechst 33342 (1:2,000 (v/v)) for 20 min. Actin filaments were stained with Phalloidin-Atto 647N (1:400 (v/v)) for 30 min. Cells were stored in PBS, kept in the dark and left at 4°C until confocal microscopy was performed with Zeiss LSM 710 and ZEN software 2010 for image processing.

Interaction of ZIKV with PEAA was analyzed by incubating different ZIKV MR766 amounts with 200 µg/ml PEAA-Rho B in an 8-well Ibidi slide for 15 min. Mixture was than fixed with 4% PFA and confocal microscopy was performed.

Antiviral activity of polyanions against clinical ZIKV isolates (ZIKV FB-GWUH-2016 and ZIKV PRVABC-59) was assessed in 30,000 HeLa cells cultured in DMEM culture medium.

The procedure was the same as indicated above for visualizing ZIKV infection of Vero E6 cells infected with ZIKV MR766.

2.2.6.2 Confocal microscopy of the uptake of Therapeutic Nucleic Acids

Uptake of therapeutic nucleic acids was investigated by seeding 150,000 A549 cells and human foreskin fibroblast (HFF) cells in different 8-well Ibidi slide with 150 µl medium. One day later, supernatant was discarded and 135 µl fresh medium (either DMEM culture medium or A549/HFF serum-free medium) was added. The media were supplemented furthermore with Hoechst 33342 (1:2000 (v/v)) to stain the nucleus and Cellmask Deep Orange plasma membrane stain 1:1,000 (v/v). Shortly before starting the measurement 15 µl of the Cy5-labelled TNA-I14 was added to the cells (20 µg/ml on cells). Image acquisition was performed with Zeiss LSM 710 every 10 min for 10 cycles. Images were processed with Fiji [302] on the Quadro P6000.

Effect of Cy5-labelled TNAs was done by seeding 40,000 A549 cells and HFF cells in different 8-well Ibidi slides in 150 µl. The next day, supernatant was discarded and 120 µl fresh medium was added. Cy5-labelled therapeutic nucleic acids were diluted 5 fold, starting with a concentration of 200 µg/ml incubated for 2 h at 37°C and subsequently, cells were infected with 15 µl with HSV-1 isolate from broncho-alveolar lavage (A549) or HSV-2 isolate from vaginal swab (HFF) with an MOI 0.02. After 72 h hours at 37°C, supernatant was discarded and cells were fixed with 4% paraformaldehyde for 10 min at 4°C. All staining steps were carried out at room temperature and between every step, cells were washed twice with PBS. Cells were blocked with blocking solution and HSV infection was visualized by staining cells infected with HSV-1 with a primary antibody against gE 1:500 (v/v) for 45 min in antibody buffer. Cells infected with HSV-2 were incubated with a primary antibody against gD 1:3,200 (v/v) for 45 min in antibody buffer. As secondary antibody Alexa Fluor 488 goat anti-mouse IgG 1:1,000 (v/v) was used in antibody buffer incubated for 45 min. Nucleus was stained with Hoechst 33342 1:2000 (v/v) for 30 min and afterwards plasma membrane was stained with Cellmask Orange plasma membrane stain 1:1,000 (v/v) for 5 min. Cells were stored in PBS, kept in the dark and left at 4°C until microscopy image acquisition using a Zeiss LSM 710 and ZEN software 2.3 blue lite (2011) for image processing.

2.2.6.3 Light microscopy imaging of HSV-1 and HSV-2 infected cells

6,000 Vero E6 cells were seeded in 96-well plate with 100 µl Vero E6 culture medium. The next day supernatant was removed and 70 µl fresh medium was added. TNAs and controls were diluted five-fold and 10 µl of 200 µg/ml was added to the cells. After 2 h of incubation at 37°C cells were infected with 20 µl of clinical isolates with an MOI of 0.02. 72 h later, Leica MC120 HD color camera connected to a microscope (Europa Promotion Leica DM IL LED, Leica microsystems) with a 10x objective was used to take pictures.

2.2.7 Statistical analysis

Statistical analyses and IC_{50} determinations were performed using GraphPad Prism version 8.0.0 for Windows. If not indicated differently, the significance level was calculated using one-way analysis of variance (ANOVA) (non-parametric, grouped), followed by Bonferroni's multiple comparison test. P values of <0.1 were considered significant (* denotes p<0.1; ** denotes p <0.01; *** denotes p <0.001; **** denotes p <0.0001).

3 Results and Discussion

3.1 Macromolecular agents as broad-spectrum antivirals against enveloped viruses - Results

Unpredicted epidemic outbreaks of viruses can lead to a huge socioeconomic burden [107,156,351,390]. Drugs that can be used for multiple viruses to prevent epidemics are still lacking. Polymers with an anionic charge possibly help to close this gap and have already been shown to inhibit infection of several viruses [188,216,306,321,326,327]. In a collaborative effort, with Alexander N. Zelikin from Aarhus University Denmark we aimed to clarify, how the structure and composition of negatively charged macromolecular drugs contribute to their antiviral activity against enveloped viruses since this group contains most of the human pathogenic viruses [88,102]. The antiviral effect of negatively charged polymers, also termed polyanions, with functional groups of carboxylate [306], phosphates/phosphonates and sulfonate [216] has already been described. This was used as a basis for the synthesis of already published and new polymers (by Alexander N. Zelikin, Aarhus University, Denmark) using Reversible Addition Fragmentation chain Transfer polymerization (RAFT). This is a controlled polymerization technique whereby the concentration ratio of monomer to RAFT agent defines the molar mass of the chain [236,424]. In this case huge batch-to-batch variations can be avoided. The monomers that were used to synthesize the polymers are summarized in Figure 4. The different functional groups are colored in red, representing the carboxylates, in green, representing the phosphates/phosphonates and in blue for polymers with functional groups of sulfonates.

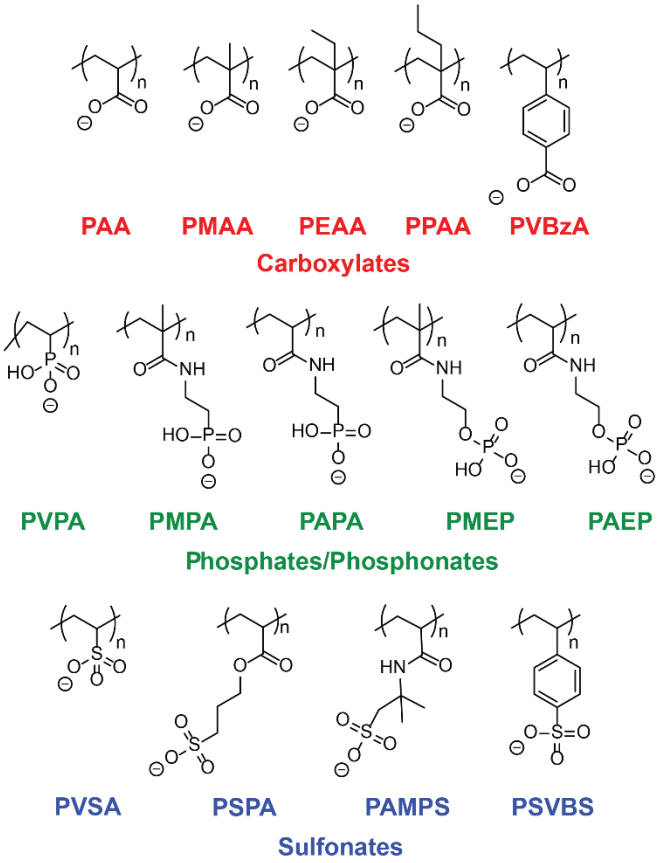

Figure 4: Chemical structures of the monomers used to synthesize respective polymers to determine the antiviral effect and the structure-activity relationship.
Carboxylates shown in red: poly(acrylic acid), PAA; poly(methacrylic acid), PMAA; poly(ethyl acrylic acid), PEAA; poly(propyl acrylic acid); PPAA, poly(vinyl benzoic acid), PVBzA. Phosphates/phosphonates shown in green: poly(vinylphosphonic acid), PVPA; poly((2-methacrylamidoethyl)phosphonic acid), PMPA; poly((2-acrylamidoethyl)phosphonic acid), PAPA; poly((2-methacrylamidoethyl)phosphate), PMEP; poly((2-acrylamidoethyl)phosphate), PAEP. Sulfonates shown in blue: poly(vinyl sulfonic acid), PVSA; poly(3-sulfopropyl acrylate), PSPA; poly(2-acrylamido-2-methyl-1-propanesulfonic acid), PAMPS; poly(sulfovinylbenzoic acid), PSVBS. Figure adapted from [300], Copyright Wiley-VCH Verlag GmbH & Co. KgaA. Reproduced with permission.

Within the group of carboxylates (PAA to PPAA) their polyanionic character was retained constant, whereas their hydrophobicity increased since the aliphatic chain gained length. Additional hydrophobic groups facilitate the interaction with protein-rich targets such as cells or viruses [20,36,169] and can be used to investigate the structure-activity relationship, irrespective of the molar mass of the polymers.

3.1.1 PEAA, PVBzA and PSVBS inhibit Zika virus infection

First, the activity of the macromolecular agents to inhibit ZIKV was assessed. For this, Vero E6 that originate from the kidney epithelium of an African green monkey [96] and the ZIKV prototype strain MR766 isolated from a sentinel rhesus macaque in Uganda were used [87]. For evaluating the antiviral effects, a colorimetric MTT assay was used as ZIKV infection causes a cytopathic effect, hence the amount of metabolic active cells can be quantified with the MTT to determine the ZIKV-induced cell death [244]. To discriminate whether the polymers act by inhibiting the viral infection, or by interacting directly with the virus, the following two different types of experiments were conducted: (1) Vero E6 cells were

incubated with the polymers prior infection or (2) the virus was pre-incubated with the polymers before cells were infected.

Two out of 14 polymers in the cell treatment (Figure 5, A) and three out of 14 polymers in the virus treatment (Figure 5, B) inhibited ZIKV induced cell death. PVBzA as well as PSVBS reduced the ZIKV induced cell death by 70-90% under both treatment conditions. Incubating the cells with PSVBS and PVBzA prior infecting them with ZIKV revealed that only the two highest concentrations (20 and 100 µg/ml) showed an antiviral effect. This was also true for PVBzA under virion treatment condition, where only 2 and 10 µg/ml reduced ZIKV induced cell death. For PSVBS already at 0.08 µg/ml a reduction of 20% in the ZIKV induced cell death was observed. In contrast to PVBzA and PSVBS, PEAA was only active under virion treatment conditions and thereby reduced the ZIKV induced cell death by 80%. Since the active concentrations of PVBzA and PSVBS were much lower under virion treatment conditions and PEAA was only active when pre-incubated with the virus these data suggest that those three polyanions directly interact with the virus.

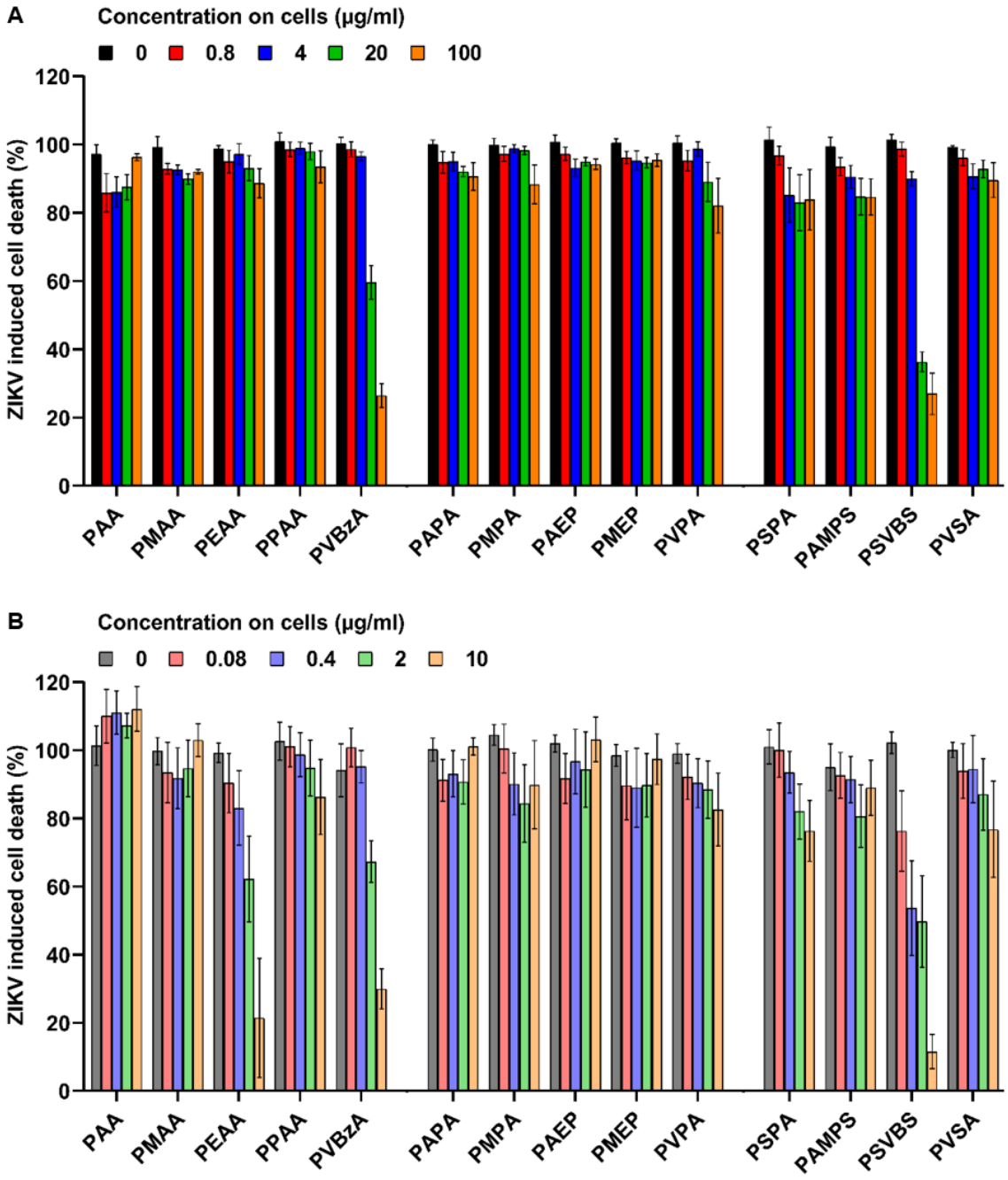

Figure 5: Identification of anionic polymers that inhibit ZIKV MR766 infection.
A) Vero E6 cells were incubated with 14 polymers in concentrations ranging from 0-100 µg/ml for 90 min prior to infection with ZIKV MR766. B) ZIKV MR766 was inoculated with 14 polymers ranging from 0-100 µg/ml for 15 min before mixtures were used to infect Vero E6 cells. ZIKV induced cell death was determined 72 h later by MTT assay. Percentage of ZIKV-induced cell death was calculated by substracting sample values from uninfected controls and normalizing to the untreated controls. Results are presented as mean ± standard error derived from two independent experiments performed in triplicates for each data point. Figure adapted from [300], Copyright Wiley-VCH Verlag GmbH & Co. KgaA. Reproduced with permission.

Since the MTT assay only represents an indirect way to measure ZIKV infection, the antiviral effect of PEAA, PVBzA and PSVBS was analysed in a more direct way. Therefore, a ZIKV immunodetection assay was used to determine the infection by assessing the viral E protein levels within the infected cells [11]. The antiviral effect of PVBzA and PSVBS under cell treatment conditions was confirmed (Figure 6, A). Furthermore, PSVBS inhibited ZIKV

infection completely at 200 µg/ml and nearly to 100% at 40 µg/ml (Figure 6, A) with a half-maximal inhibitory concentration (IC_{50}) of 14 µg/ml. PVBzA inhibited the ZIKV infection only to around 50%. This effect is not as pronounced as with the detection of ZIKV induced cell death where even 20 µg/ml revealed a 40% reduction (Figure 5, A). Under virus treatment conditions the effect of PEAA, PVBzA and PSVBS was confirmed (Figure 6, B). At the highest concentration, PEAA revealed a 90% reduction of ZIKV infection with an IC_{50} of 4.1 µg/ml and PSVBS inhibited ZIKV infection completely with an IC_{50} of 1.4 µg/ml. The metabolic activity of the cells in presence of the polymers was not affected suggesting that the polymers are not toxic (Figure 6, C). These data support that the antiviral effect is not a result of cytotoxic effects of the polymers and the inhibition is due to a direct interaction of the polyanions with the virus.

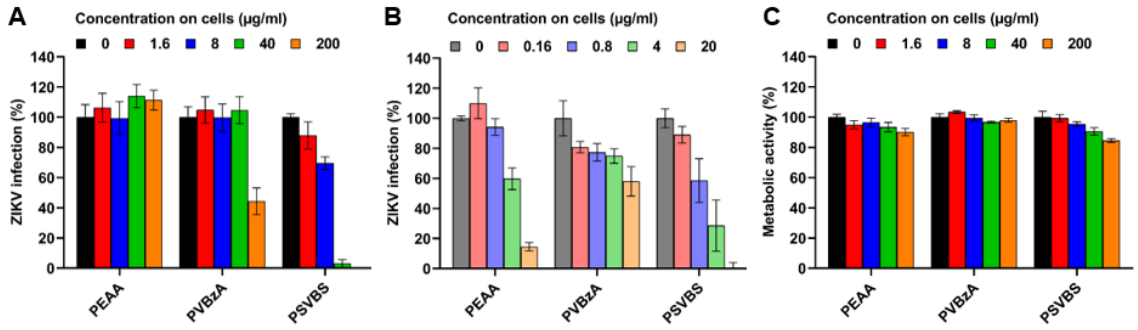

Figure 6: PEAA, PVBzA and PSVBS inhibit ZIKV without any cytotoxic effects on Vero E6 cells.
A) Vero E6 cells were inoculated with PEAA, PVBzA or PSVBS 90 min prior infection with ZIKV MR766. B) ZIKV MR766 was inoculated with PEAA, PVBzA or PSVBS 15 min prior mixtures were used to infectVero E6 cells. The infection was quantified 36 h post infection by virus immunodetection assay using the flavivirus antibody 4G2. Data are normalized to infection in the absence of the respective polymer. C) Vero E6 cells were exposed to the three polymers and cell viability was assessed by MTT assay 72 h later. Metabolic activity was normalized to untreated control. Results are presented as mean ± standard error derived from two independent experiments performed in triplicate for each data point. Figure adapted from [300], Copyright Wiley-VCH Verlag GmbH & Co. KgaA. Reproduced with permission.

To take a closer look into the antiviral effect of the three identified ZIKV inhibiting polymers, I performed confocal laser scanning microscopy (LSM). To avoid strong cytopathic effects caused by ZIKV a low viral dose was used to allow LSM. As already shown in the previous experiment the infection with ZIKV can also be assessed on the viral E protein levels. To visualize the expression of E protein a secondary Alexa Flour 488® goat anti-mouse IgG was used (green channel, Figure 7). A dose-dependent reduction of ZIKV E protein is observed, whereby no expression of ZIKV E protein was detected at 20 µg/ml revealing that the viral infection was completely inhibited. Cellular morphology as well as staining of the nuclei (Figure 7, blue) and cytoskeleton (Figure 7, red) were similar for uninfected and PEAA-exposed cells, excluding cytotoxic effects, which confirms the results of the preserved metabolic activity (Figure 6, C).

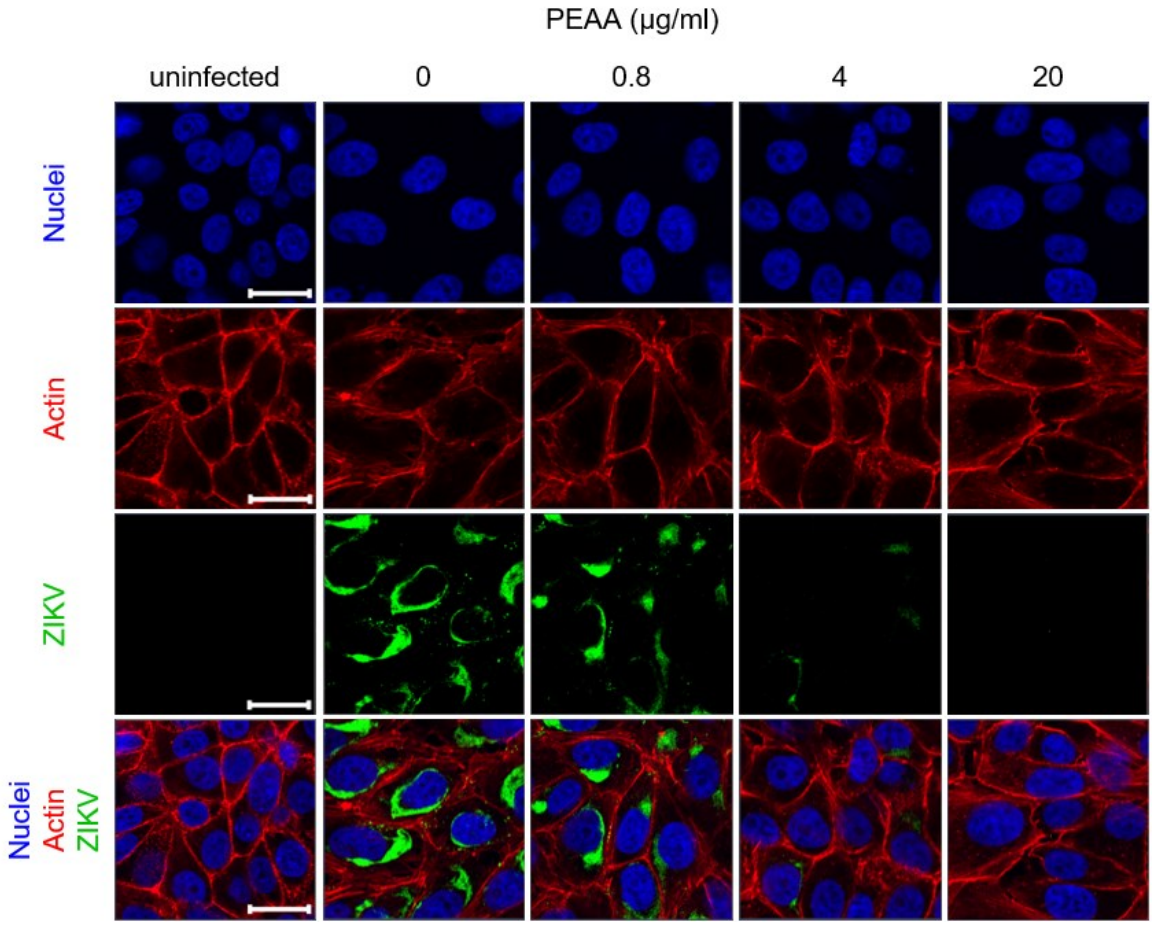

Figure 7: PEAA inhibits ZIKV MR766 infection in Vero E cells under virion treatment conditions.
ZIKV MR766 was inoculated with PEAA 15 min prior mixture was used to infect Vero E6 cells. 72 h later cells were fixed and stained for ZIKV protein E (green), nuclei (blue) and actin (red). Shown are exemplary confocal images. Scale bars correspond to 20 µm (same for all panels). Figure adapted from [300], Copyright Wiley-VCH Verlag GmbH & Co. KgaA. Reproduced with permission.

The same was done for PVBzA and PSVBS, whereby the cells were pre-incubated with the compounds prior infection. Exemplary, the merged channels of the LSM images are shown in Figure 8 (single channels are shown in Supplementary figure 1 and Supplementary figure 2, Appendix). Although at concentrations of 40 µg/ml and 200 µg/ml of PVBzA less ZIKV E protein was expressed, a complete reduction of the viral E protein levels was not achieved (Figure 8, A), confirming the previous results obtained with the ZIKV immunodetection assay (Figure 6, A). PSVBS also showed a dose-dependent reduction of the ZIKV E protein. At 40 and 200 µg/ml no ZIKV E expression was observed (Figure 8, B), revealing that PSVBS was able to inhibit the infection completely, confirming the previous result (Figure 6, A). Neither PVBzA nor PSVBS caused morphological changes of the cells with similar cell nuclei and cytoskeleton appearance for uninfected and PVBzA/PSVBS treated cells (Figure 8), which was already shown by determination of the metabolic activity (Figure 6, C).

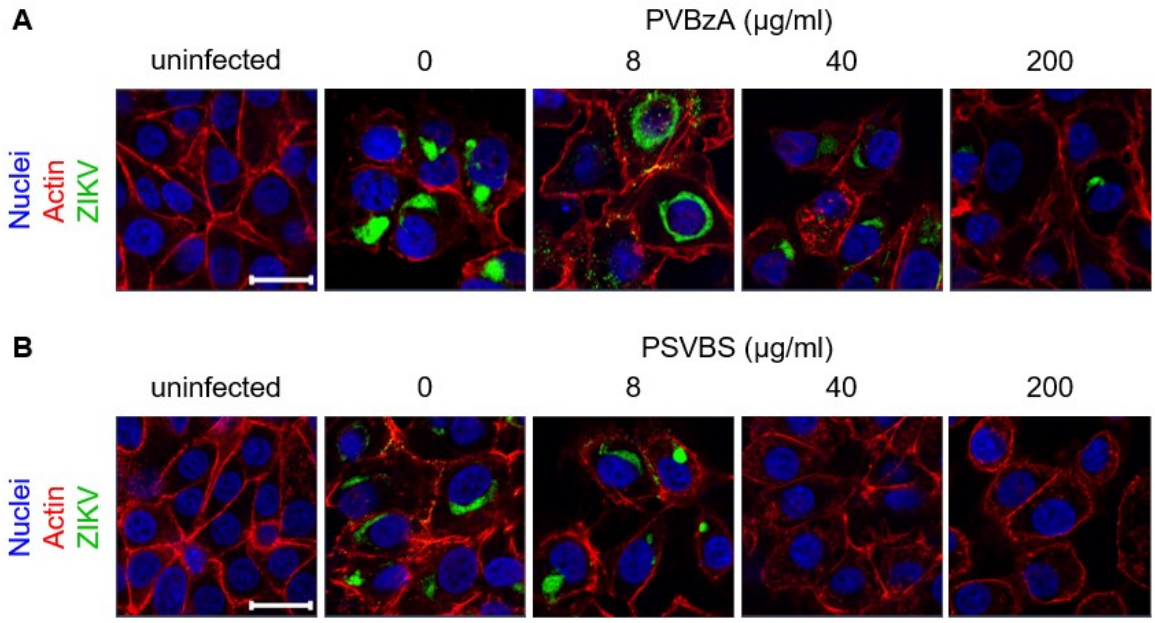

Figure 8: PVBzA and PSVBS inhibit ZIKV MR766 infection in Vero E6 cells under cell treatment conditions. Vero E6 cells were incubated with A) PVBzA or B) PSVBS for 90 min prior infection with ZIKV MR766. 72 h later cells were fixed and stained for ZIKV protein E (green), nuclei (blue) and actin (red). Shown are exemplary merged confocal images. Scale bars correspond to 20 µm (same for all panels). Figure adapted from [300], Copyright Wiley-VCH Verlag GmbH & Co. KgaA. Reproduced with permission.

3.1.2 PEAA clusters Zika virus and inhibits viral attachment

To further clarify the mechanism underlying the direct acting PEAA (Figure 5, Figure 6 A, B) I studied the interaction of the virus with PEAA using fluorescence activated cell sorting (FACS) and LSM. Therefore, PEAA was fluorescently labelled with either Fluorescein Isothiocyanate (FITC) or Rhodamine B (Rho B) (done by Alexander N. Zelikin, Aarhus University, Denmark). PEAA-Rho B and PEAA-FITC were mixed with different virus concentrations prior analysis. PEAA-Rho B without addition of ZIKV cannot be seen in the LSM (Figure 9, A). Up to a 625-fold dilution of ZIKV yellow clusters appeared (Figure 9, A), which indicate that PEAA seems to capture the virions and thereby inhibiting the infection. The clustering was confirmed with FITC labelled PEAA in FACS. The Mean Fluorescence Intensity (MFI) was increased with undiluted and 5-fold diluted ZIKV (Figure 9, B).

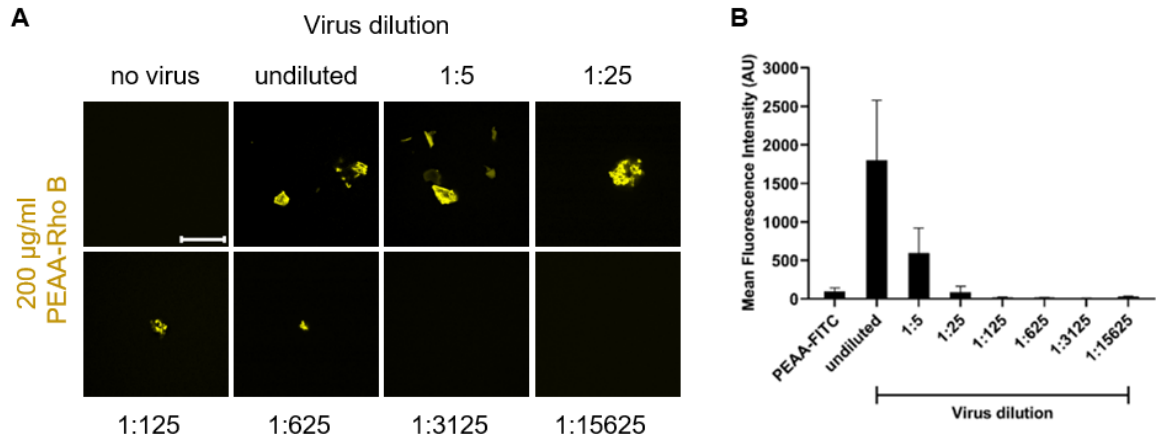

Figure 9: Clustering of ZIKV by fluorescently labelled PEAA.
A) ZIKV MR766 was diluted in a 5-fold series and incubated with 200 µg/ml PEAA-Rhodamine B in absence of cells for 15 min. PEAA-Rhodamine B was also incubated with buffer (no virus) in absence of cells for 15 min. Formed cluster were imaged by LSM. B) ZIKV MR766 was diluted in a 5-fold series and incubated with 200 µg/ml of PEAA-FITC in absence of cells for 15 min. PEAA-FITC was also incubated with buffer (no virus) in absence of cells for 15 min. Mean Fluorescence Intensity was measured by FACS. Scale bar corresponds to 20 µm (same for all panels). Figure adapted from [300], Copyright Wiley-VCH Verlag GmbH & Co. KgaA. Reproduced with permission.

Since the fluorescence labelling may also affect the properties of the polyanion, I investigated the antiviral effect of PEAA-Rho B under virion treatment conditions using LSM. With increasing concentration of PEAA-Rho B, decreasing fluorescence signal of ZIKV E protein was detected in cells using LSM (Figure 10, detailed images Supplementary figure 3, appendix). Furthermore, distinct clusters of PEAA-Rho B, possibly interacting with ZIKV, are visible at concentrations of 4 and 20 µg/ml PEAA-Rho B. The polymer is also distributed over the cells which can be a result of soluble, non-virus interacting polymer (Figure 10). PEAA-Rho B does not have an impact on the cell viability since the morphology of the cells is not changed and nuclei and cytoskeleton staining for uninfected and PEAA-Rho B exposed cells was similar (Figure 10). This indicates that the properties of PEAA of clustering ZIKV were unaffected by the addition of a fluorescence label and infection was still prevented.

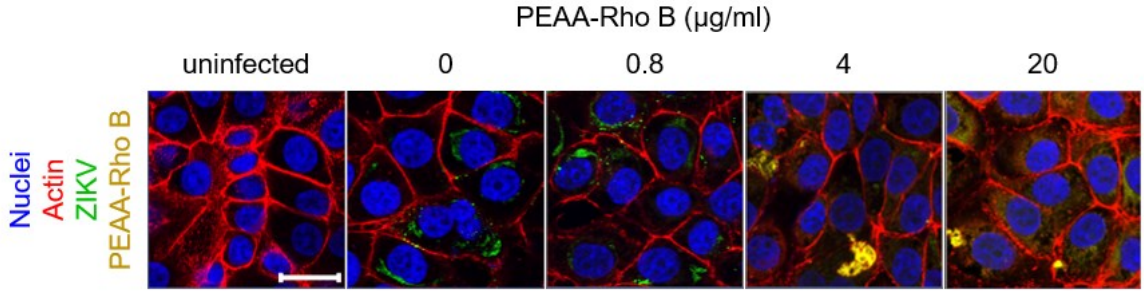

Figure 10: PEAA-Rho B inhibits ZIKV MR766 under virion treatment conditions.
MR766 was inoculated with PEAA-Rho B for 15 min prior mixture was used to infect Vero E6 cells. 72 h later cells were fixed and stained for ZIKV protein E (green), nuclei (blue) and actin (red). Shown are exemplary confocal images. Scale bars correspond to 20 µm (same for all panels). Figure adapted from [300], Copyright Wiley-VCH Verlag GmbH & Co. KgaA. Reproduced with permission.

3.1.3 PEAA, PVBzA and PSVBS inhibit clinical relevant ZIKV strains in HeLa cells

All previous performed experiments were conducted with the prototype virus ZIKV MR766 from 1947 1947 [87]. To analyze the effect of the lead polymers PEAA, PVBzA and PSVBS on clinically relevant ZIKV isolates two different strains were used to infect human cervical carcinoma (HeLa) cells. ZIKV FB-GWUH-2016 was isolated from the brain of an aborted fetus suffering from microcephaly in 2016 [91] and PRVABC59 originated from serum of an infected patient from Puerto Rico in 2015 [193].

LSM of ZIKV FB-GWUH-2016 or PRVABC59 pre-incubated with PEAA revealed that at 20 µg/ml no ZIKV E protein was expressed anymore (Figure 11, A, detailed images Supplementary figure 4, appendix). HeLa cells were also pre-incubated with PVBzA or PSVBS and afterwards either infected with ZIKV FB-GWUH-2016 or PRVABC59. Expression of ZIKV E protein of FB-GWUH-2016 is lower at concentrations of 40 and 200 µg/ml (Figure 11, B, detailed images Supplementary figure 5, appendix). Nevertheless, the infection was not inhibited completely. However, this is true for 200 µg/ml PVBzA and PRVABC59, where no ZIKV E protein was detected anymore. PSVBS inhibited infection with FB-GWUH-2016 at concentrations of 40 and 200 µg/ml completely, whereas this was only the case at a concentration of 200 µg/ml when HeLa cells were infected with PRVABC59 (Figure 11, C, detailed images Supplementary figure 6, appendix). Neither the nuclei nor the cytoskeleton (Figure 11, blue and red stains) are affected by the polymers excluding possible toxic effects on the HeLa cells. The results are in line with the previous results on the antiviral activity of the three polymers on ZIKV MR766, which means that not only the prototype virus strain inhibited, but also clinical relevant isolates.

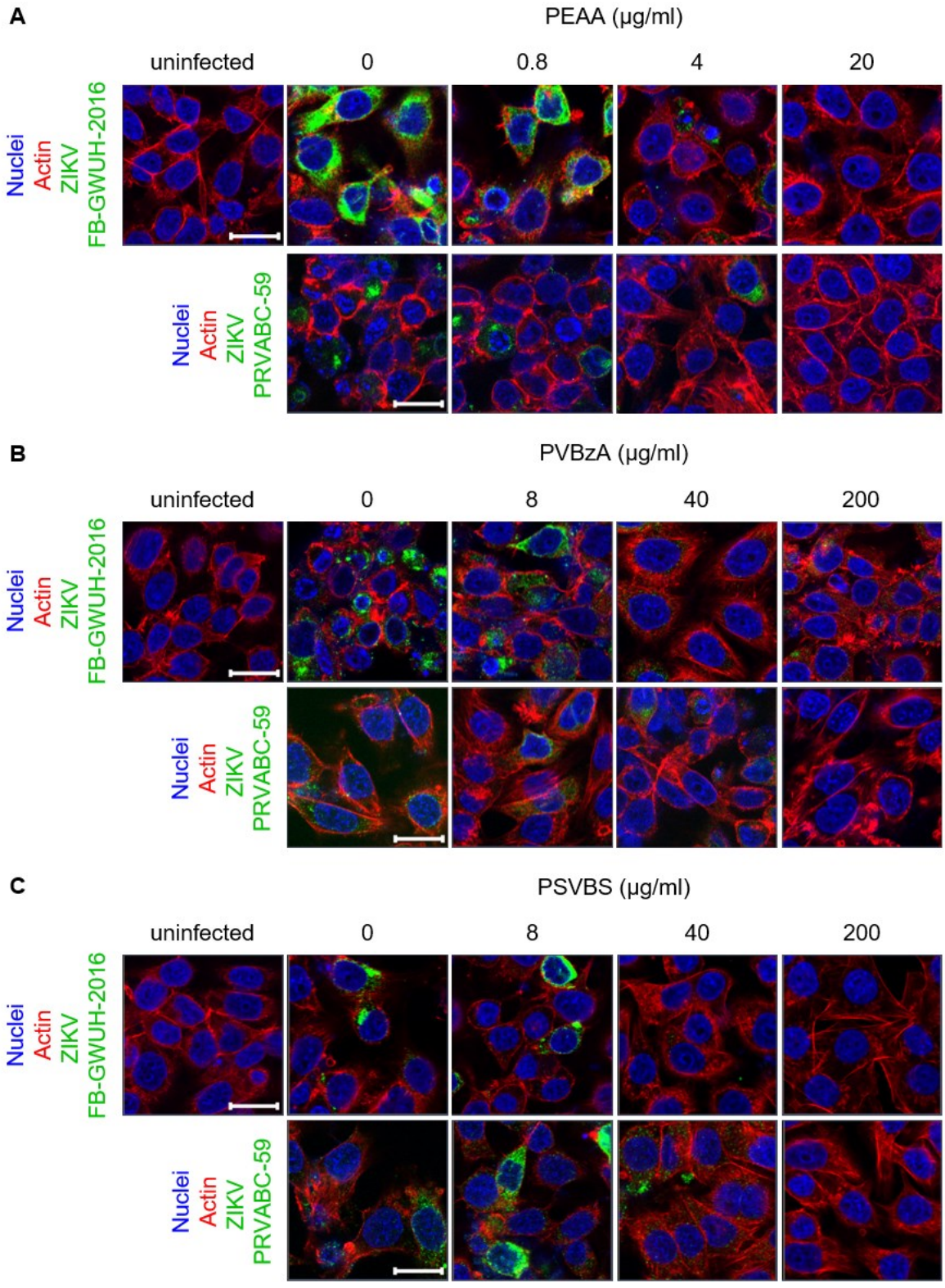

Figure 11: PEAA, PVBzA and PSVBS inhibit clinically relevant ZIKV isolates in HeLa cells
FB-GWUH-2016 or PRVABC-59 were incubated with PEAA for 15 min prior mixture was used to infect HeLa cells. B) HeLa cells were incubated with PVBzA for 90 min prior infection with FB-GWUH-2016 or PRVABC-59. C) HeLa cells were incubated with PSVBS for 90 min prior infection with FB-GWUH-2016 or PRVABC-59. 72 h later cells were fixed and stained for ZIKV protein E (green), nuclei (blue) and actin (red). Shown are exemplary confocal images. Scale bars correspond to 20 µm (same for all panels). Figure adapted from [300], Copyright Wiley-VCH Verlag GmbH & Co. KgaA. Reproduced with permission.

3.1.4 Polyanions exhibit broad antiviral activity against enveloped viruses

Since 3 out of 14 tested polymers displayed antiviral activity against ZIKV the question remained, whether also other viral pathogens from the group of enveloped viruses are affected.

First, I tested the possible cytotoxic effects of the polymers on the different cell lines used in the infection assays. As positive control heparin was used. This is polyanion has been described earlier to inhibit several viruses by impeding the viral entry [13,216,307], the proposed mechanism of action for the polyanions that were able to inhibit ZIKV infection. None of the tested polymers had an influence on the metabolic activity of each cell line (Figure 12), which means that possible antiviral effects do not result from cytotoxic effects of the polymers.

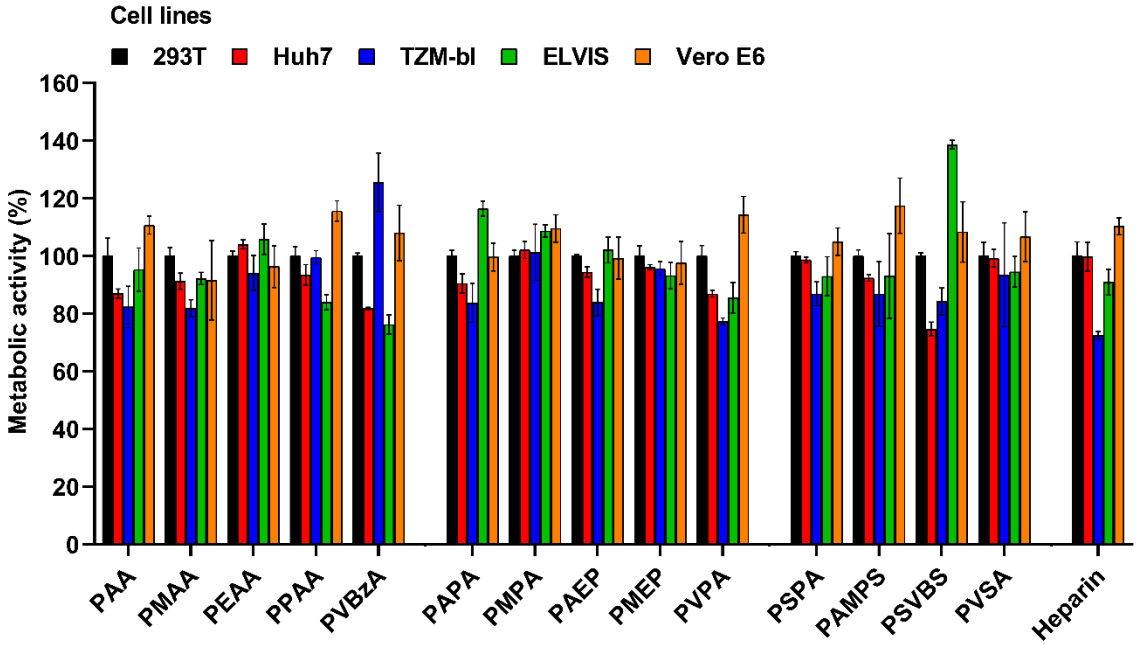

Figure 12: Determination of the cytotoxic effect of polyanions on 5 different cell lines used for different virus or lentiviral pseudotypes.
Indicated cell lines were incubated for 3 days with the highest concentration (50 µg/ml) of the different polyanions used in the respective antiviral screens (see **Figure 13** - **Figure 15**). Metabolic activity of cells was assessed by MTT and was normalized to untreated control. Results are presented as mean ± standard error derived from biological triplicates for each data point. Figure adapted from [300], Copyright Wiley-VCH Verlag GmbH & Co. KgaA. Reproduced with permission.

First, the antiviral effect of the polymers against HIV type 1 was tested. Worldwide 36.7 million people are currently infected with HIV-1 [360], which was discovered in 1983 [17,62] and is one of the causative agents for the acquired immunodeficiency syndrome (AIDS) [132,226]. For evaluation of the infection inhibiting potential of the polymers, either TZM-bl cells or HIV-1 was pre-incubated with the different polymers. Under cell treatment condition 12 out of 14 polymers were able to inhibit HIV-1 infection dose-dependently (Figure 13, A). Under virion treatment conditions 10 out of 14 polymers were able to dose-dependently reduce infection (Figure 13, B). The effect of the polymers on the viruses was much more pronounced when virions instead of cells were incubated with the polymers which results in much lower IC_{50} values, summarized in Table 2. PSPA, PAMPS, PSVBS

and PVSA, which belong to the group of sulfonates were able to inhibit HIV-1 with IC_{50} values in the low ng/ml range (Table 2). Also the control heparin inhibited HIV-1 dose-dependently. Heparin is known to inhibit the viral entry which supports the assumption that also the polyanions directly interact with viruses as shown by the clustering of ZIKV by PEAA (Figure 9). PPAA (carboxylate), PAEP and PAPA (phosphates/phosphonates) showed the lowest inhibitory effect on HIV-1 indicating that structural differences of the polymers contribute to the antiviral effect, since all sulfonates were able to inhibit HIV-1 infection.

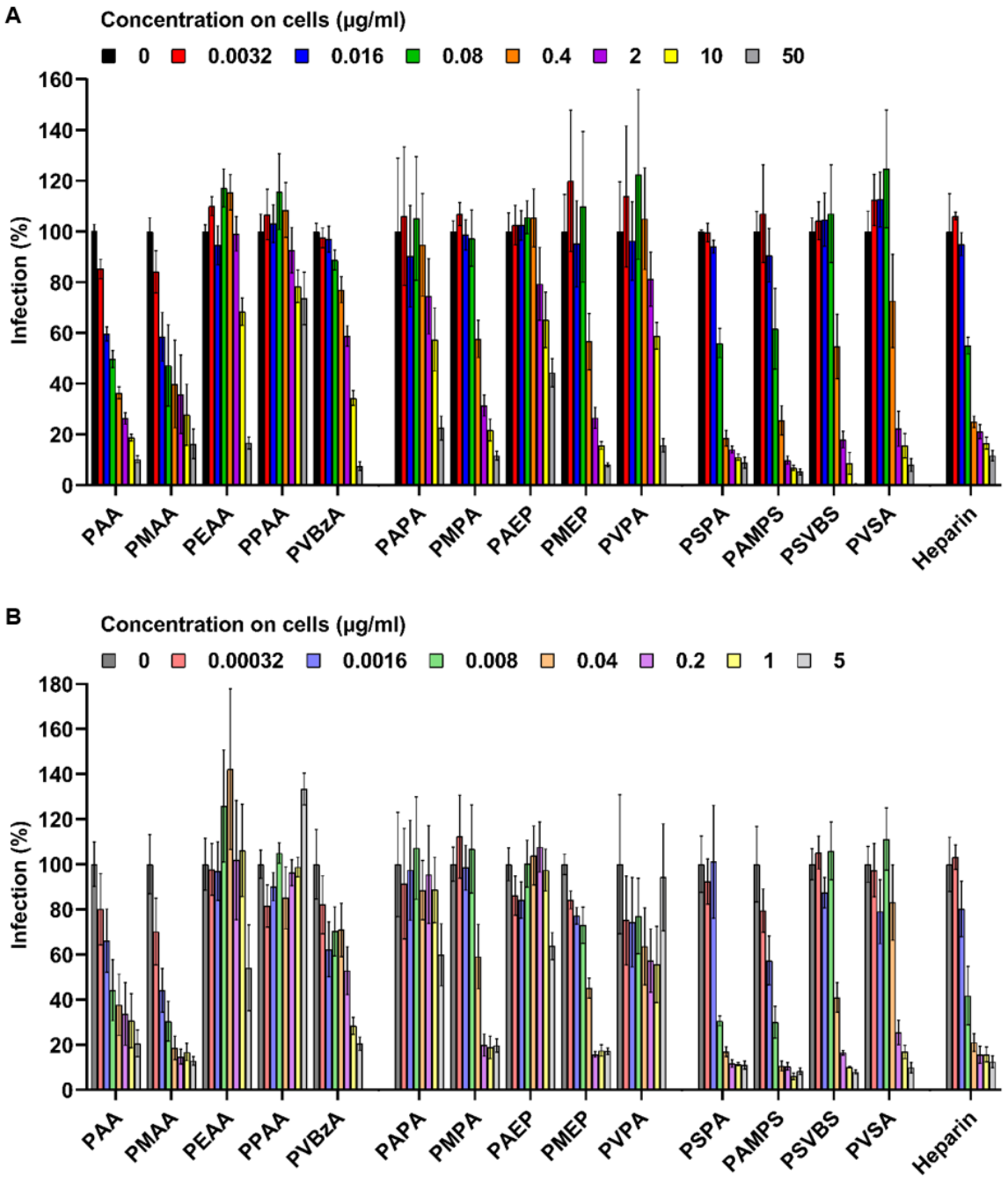

Figure 13: Identification of anionic polymers that inhibit HIV-1 infection.
A) TZM-bl cells were incubated with the 14 polymers or Heparin as control for 60 min prior infection with HIV-1. B) HIV-1 was inoculated with the 14 polymers or Heparin as control 10 min prior mixtures were used to infect TZM-bl cells. Infection was determined after 72 h by quantifying β-galactosidase activities of whole cell lysates. Data are normalized to infection in the absence of the respective polymer. Results are presented as mean ± standard error derived from two independent experiments performed in triplicates for each data point. Figure adapted from [300], Copyright Wiley-VCH Verlag GmbH & Co. KgaA. Reproduced with permission.

Next, I determined the antiviral activity of the polymers against HSV-2. Under cell treatment conditions 5 out of 14 polyanions were able to dose-dependently inhibit HSV-2 infection (Figure 14, A). Under virion treatment conditions 4 out of 14 polymers reduced the HSV-2 infection in a dose-dependent manner (Figure 14, B). As already seen in the identification of polyanions that inhibit HIV-1 infection (Figure 13) the polymers with a sulfonate group, namely PSPA, PAMPS, PSVBS and PVSA, were the ones that also distinctly inhibited HSV-2. In the case of HSV-2 this resulted in low µg/ml IC_{50} values (Table 2). It is known that HSV-2 uses heparan sulfate, to initiate viral attachment. This supports the notion that polymers possible inhibit infection by interaction with the virus. In the group of carboxylates, only PMAA and PVBzA inhibited HSV-2. Under cell treatment conditions the IC_{50} of PMAA is 26 µg/ml and for PVBzA 4.8 µg/ml. Under virion treatment conditions PMAA had an IC_{50} of 0.5 µg/ml and PVBzA of 0.85 µg/ml. In the group of phosphates/phosphonates only PVPA showed antiviral activity with an IC_{50} value of 14.2 under cell treatment conditions.

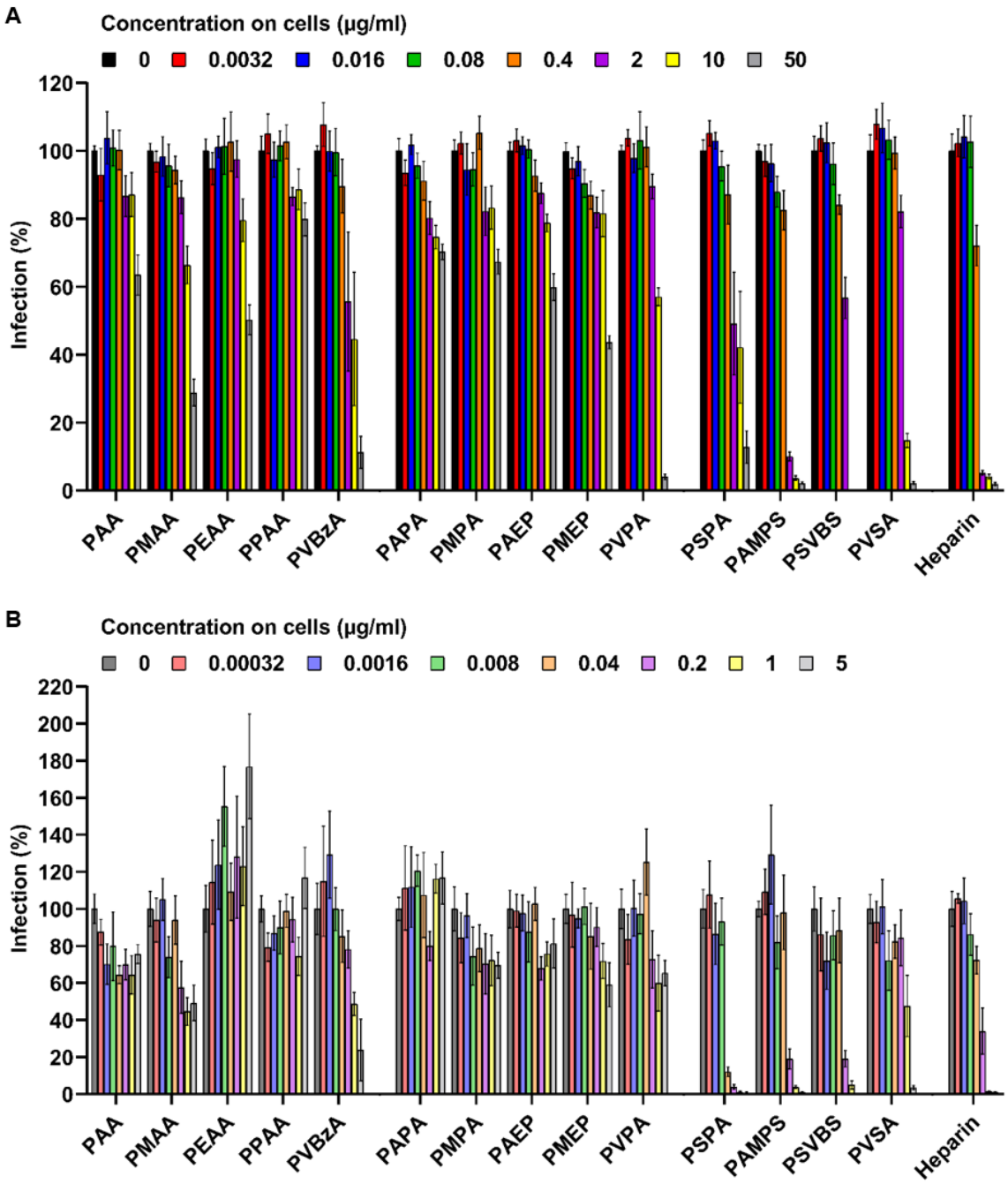

Figure 14: Identification of anionic polymers that inhibit HSV-2 infection under cell treatment conditions.
A) ELVIS™ cells were incubated with the 14 polymers or Heparin as control for 60 min prior infection with HSV-2. B) HSV-2 was inoculated with the 14 polymers or Heparin as control 10 min prior mixtures were used to infect ELVIS™ cells. Infection was determined after 36 h by quantifying β-galactosidase activities of whole cell lysates. Data are normalized to infection in the absence of the respective polymer. Results are presented as mean ± standard error derived from two independent experiments performed in triplicates for each data point. Figure adapted from [300], Copyright Wiley-VCH Verlag GmbH & Co. KgaA. Reproduced with permission.

Table 2: IC_{50} as representation for the antiviral activity of polymers against HIV-1 and HSV-2.
The IC_{50} values were calculated based on the final cell culture concentration of the polyanions, which was 10 times lower under virion treatment conditions. Table adapted from [300], Copyright Wiley-VCH Verlag GmbH & Co. KgaA. Reproduced with permission.

	HIV-1		HSV-2	
Polymer	IC_{50}, cell treatment (µg/ml)	IC_{50}, virus treatment (µg/ml)	IC_{50}, cell treatment (µg/ml)	IC_{50}, virus treatment (µg/ml)
PAA	0.1	0.005	>50	>5
PMAA	0.05	0.001	26	0.5
PEAA	23	>5	>50	>5
PPAA	>50	>5	>50	>5
PVBzA	4	0.25	4.8	0.85
PAPA	18	>5	>50	>5
PMPA	0.7	0.07	>50	>5
PAEP	38.5	>5	>50	>5
PMEP	0.6	0.03	44	>5
PVPA	16.5	>5	14.2	>5
PSPA	0.12	0.003	2	0.02
PAMPS	0.15	0.002	0.83	0.11
PSVBS	0.4	0.04	2.6	0.08
PVSA	0.8	0.09	4.3	0.9

We next tested the compounds against pseudotyped viral particles (pp). In this case glycoproteins of different viruses are incorporated into a lentiviral luciferase encoding virus to allow studying of the entry of viruses that normally only can be analyzed in a laboratory with biosafety level 4. An overview of the glycoproteins and the corresponding viral characteristic are listed in Table 3.

Table 3: Glycoproteins from different viruses used for the production of pseudotyped lentiviral particles .

Origin of glycoproteins	Family	Characteristics
(European bat) lyssa virus	*Rhabdoviridae*	Enveloped; cause rabies in humans and animals.
Rabies virus		
Ebola virus	*Filoviridae*	Enveloped; responsible for sporadic outbreak in Africa accompanied with fatal hemorrhagic fevers.
Marburg virus		
SARS coronavirus	*Coronaviridae*	Enveloped; cause of SARS epidemic in 2002.
Lassa virus	*Arenaviridae*	Enveloped; causes Lassa fever.
Influenza A virus	*Orthomyxoviridae*	Enveloped; avian influenza/bird flu.

In this approach the antiviral activity was only assessed under cell treatment conditions. The above gained results showed polymers active under cell treatment conditions, are also active under virion treatment conditions. The only exception is ZIKV were PEAA was only active in virion treatment. For influenza pp only PVPA from the group of phosphates/phosphonates reduced the infection by more than 80% (Figure 15, G). For rabies pp (Figure 15, B), Ebola pp (Figure 15, C), SARS pp and lassa pp (Figure 15, E, F) two of the tested polymers were able to inhibit the infection by at least 80%. For lyssa pp (Figure 15, A) 3 of 14 polymers, and for Marburg pp (Figure 15, D) 6 of 14 polymers reduced to infection by at least 80%. Striking is the activity of PVBzA from the group of carboxylates. This polymer was able to inhibit all viral pseudoparticles for at least 50% (Figure 15). In Lyssa pp (Figure 15, A), SARS pp (Figure 15, E) and Lassa pp (Figure 15, F) the effect is very pronounced, with a

reduction of infection of more than 90%. This is followed by PEAA which was especially active against Ebola pp (Figure 15, C) and Marburg pp (Figure 15, D). Polymers in the group of phosphates/phosphonates showed only mild inhibitory effects, except for PVPA which was especially active against Marburg pp (Figure 15, D), SARS (Figure 15, E) as well as influenza pp (Figure 15, G). Within the group of sulfonates PSVBS was the only one that inhibited 6 out of 7 pseudotyped viral particles.

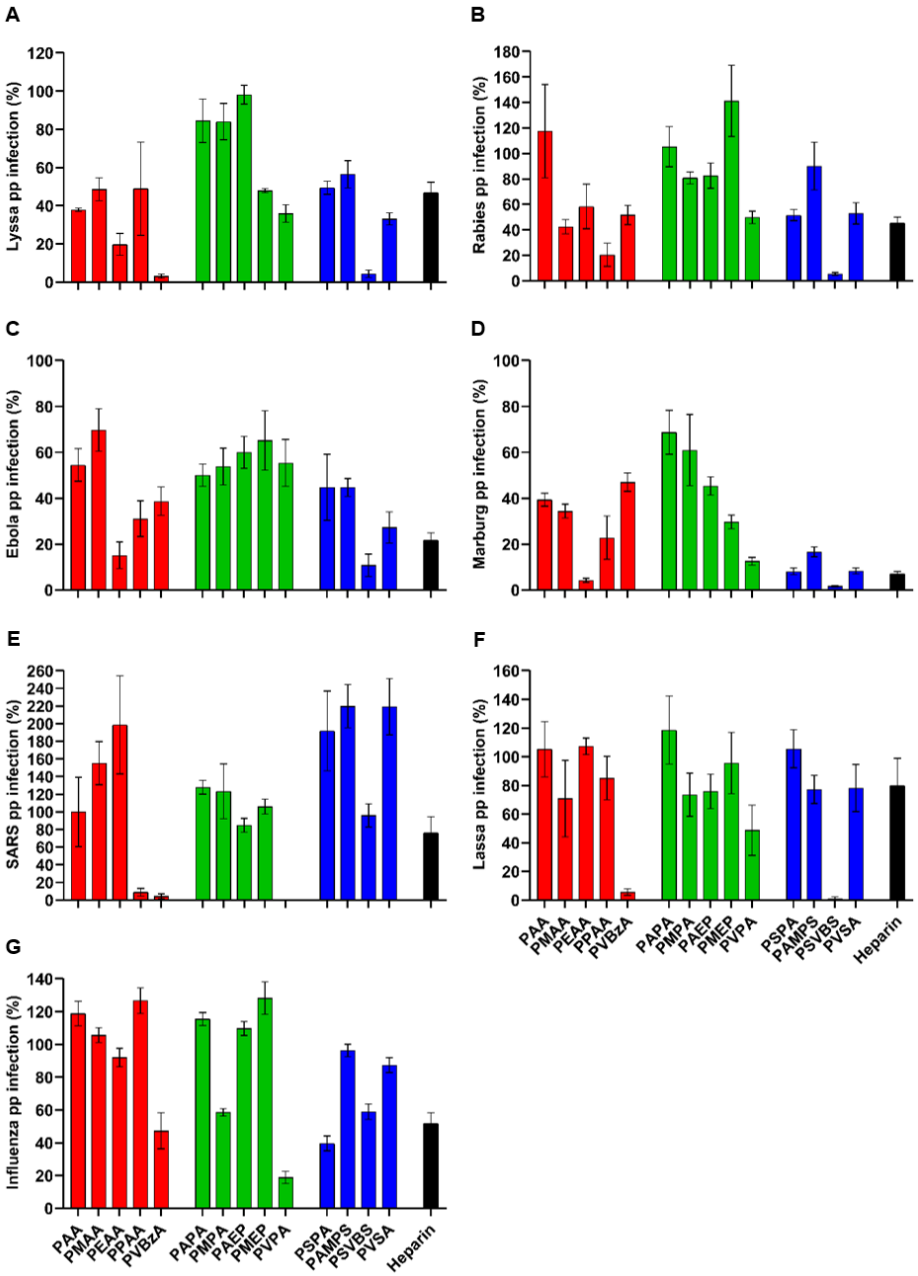

Figure 15: Identification of anionic polymers that inhibit lentiviral pseudotypes carrying different glycoproteins under cell treatment conditions.
Lenntiviral pseudotypes carrying different glycoproteins derived from A) European bat lyssa virus, B) Rabies virus, C) Ebola virus, D) Marburg virus, E) SARS virus, F) Lassa fever virus, G) influenza virus. Huh7 cells (forlentiviral pseudotypes of European bat lyssa virus, Rabies virus, Ebola virus, Marburg virus and SARS virus), Vero E6 cells (for lentiviral pseudotypes of Lassa virus) and 293T cells (for lentiviral pseudotypes of influenza virus) were exposed to 50 µg/ml of the polymers or Heparin as control and then infected with the diffent lentiviral pseudoparticles. Infection was determined 72 h post infection by quantifying luciferase activities in whole cellular lysates. Data are normalized to infection in the absence of the respective polymer. Results are presented as mean ± standard error derived from two independent experiments performed in triplicates for each data point. Figure adapted from [300], Copyright Wiley-VCH Verlag GmbH & Co. KgaA. Reproduced with permission.

To test whether only enveloped viruses are targeted by the tested polymers a non-enveloped Adenovirus (HAdV5) was chosen. None of the tested polymers inhibited the non-enveloped adenovirus (Figure 16).

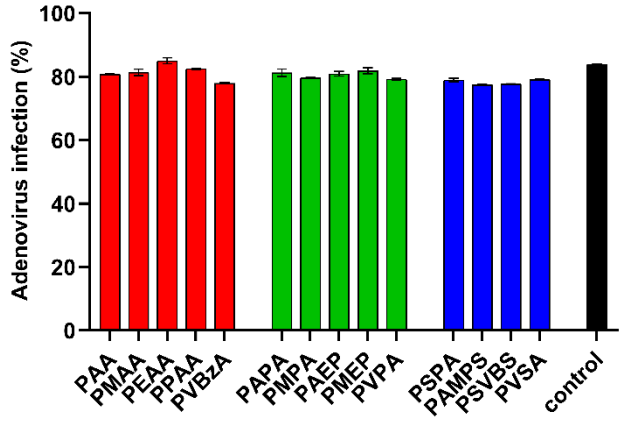

Figure 16: Anionic polymers do not have an effect on Adenovirus infection.
A549 cells were treated with 50 µg/ml of the polymers for 10 min prior infection with Ad5-EGFP. Infection rates were determined 24 h later by flow cytometry. Data are normalized to infection in absence of the respective polymer. Values are shown as the mean ± standard error derived from duplicate infections. Assay was performed by Florian Kreppel, Institue of Gene Therapy, Ulm University Medical Center. Figure adapted from [300], Copyright Wiley-VCH Verlag GmbH & Co. KgaA. Reproduced with permission.

To highlight the effect of the polymers, their maximum activity against the various analyzed viruses are summarized in Figure 17. Phosphates/phosphonates are the least antivirally active tested polymers. Intermediate antiviral activity is represented by the group of carboxylates. The most active polymer from this group is PVBzA. It was the only polymer from the carboxylates that inhibited all tested viruses, although with moderate inhibition rates. The group of sulfonates includes the most effective antiviral agent that inhibited the infection of many tested viruses and pseudotyped viral particles from 70 to more than 99%. The most active polymer in this group was PSVBS.

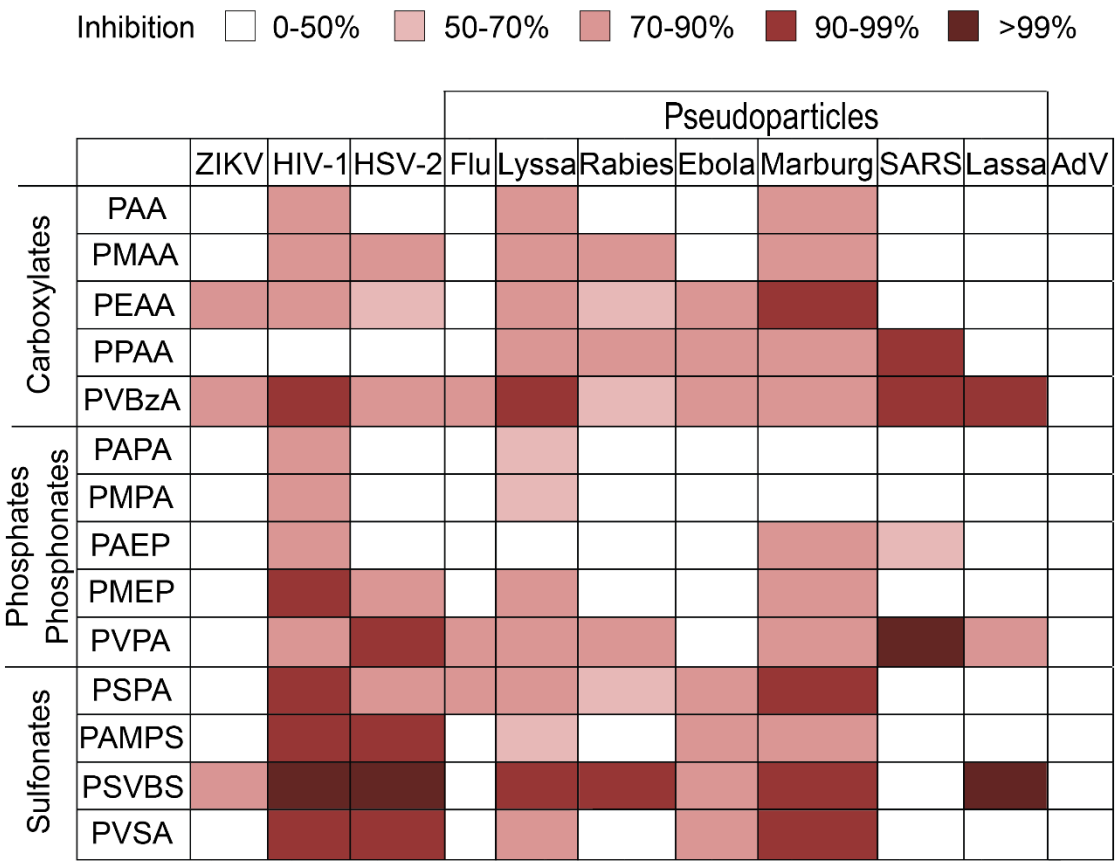

Figure 17: Summary of the maximum antiviral activities of anionic polymers against the different viruses.
The shade of the colour represents the antiviral effect, colourcoded as indicated. Lentiviral pseudotype of influenza (Flu). Figure adapted from [300], Copyright Wiley-VCH Verlag GmbH & Co. KgaA. Reproduced with permission.

3.2 New approaches for the design of broad-spectrum antiviral agents - Discussion

Viruses can have a great socioeconomic burden. Some are associated with high mortality rates such as Ebola or Influenza, others are associated with high morbidity such as HSV or HIV. In many cases drugs to treat viruses are still missing and they are often invented after the virus became epidemic or pandemic [69,127,181,371]. Furthermore, treatment strategies like they are used for bacteria such as the usage of one drug that can treat multiple pathogens are lacking for viruses [24]. Most viruses that can infect and being pathogenic to human belong to the group of enveloped viruses [88]. Polymers with different functional groups such a carboxylate, e.g. PMAA, phosphate/phosphonate or sulfate, e.g. heparin are negatively charged and already have been described as attachment inhibitors for several viruses [216,306,326,327]. Until now, structure related studies based on their antiviral effects are missing, which was the focus of my study.

A total of 14 anionic polymers were tested against 3 viruses and 7 pseudotyped lentiviral particles, which are all enveloped. Additionally, one non-enveloped virus was included. For all tested viruses the least active polymers derived from the group of phosphates/phosphonates. In general, phosphoric residues have a relatively high pKa [42,422]. The higher the pKa value the lower is the dissociation ability at physiological pH and the less anionic the residues will be. PVPA was the most active polyanion from the group of phosphates/phosphonates and inhibited 8 out of 11 viruses. For the other polymers from this group it seems to be the case that also the hydrophobicity of the aliphatic chain contributes to the antiviral activity. PMEP and PAEP theoretically have a higher pKa compared to PMPA and PAPA [42,422] and in this row PMEP, with a longer aliphatic chain inhibited the most (4 out of 11) viruses.

The group of carboxylates was able to inhibit several viruses. PAA inhibited 3 viruses, PMAA inhibited 5 viruses and PEAA was able to inhibit 7 viruses. Somehow, PPAA only inhibited 5 viruses, which was not expected since in the row from PAA to PPAA the anionic character of the polymers was constant. Only the hydrophobicity increased since the length of the aliphatic chain was extended. However, PVBzA was able to inhibit all enveloped viruses. It differs from the other carboxylates since it has a styrolic group, which is rather hydrophobic. This supports the notion that both anionic nature and hydrophobicity of the polymers define the antiviral activity.

The group of sulfonates made up polymers that showed the broadest antiviral effect. At least 5 viruses were inhibited by each sulfonate and the inhibition was at least 70%. PSVBS inhibited 8 out of 11 viruses and showed inhibition rates of over 99% in 3 out of the 8 viruses. Interestingly, PSVBS share structure similarities with PVBzA from the carboxylates. Both have a hydrophobic styrolic group and this further highlights that antiviral activity is a result of negative charge as well as hydrophobicity of the polymers.

The interaction of hydrophobic groups with protein-rich targets such as cells or viruses has already been shown [33,36,153,196,355]. Analysis with confocal laser scanning microscopy and FACS showed that PEAA assembled ZIKV thereby preventing the infection. This might be a result of physical interactions between PEAA and hydrophobic membrane as well as

glycoproteins of ZIKV [33,153,196]. This was underlined by the observation that the polymers inhibited the viruses at lower concentration when the viruses were pre-incubated with the polymers. Furthermore, sulfonates, to which heparin also belongs to have already been described to inhibit HSV infection by interacting through hydrophobic binding with the virus [310,357,402]. The glycoproteins B and/or C of HSV mediate viral attachment through contact with cellular glycosaminoglycans, especially heparan sulfate. Heparin therefore interferes with those glycoproteins thereby preventing infection [123,143,357]. This is also in agreement with the amount of glycoproteins on the viral surface. HIV-1, with the least amount of glycoproteins of 7-15 [60,61,199,419,420] was inhibited by 13 out of 14 polymers. HSV-2, with around 700 glycoproteins on the virus [137] was inhibited by 9 out 14 polymers. The densely packed ZIKV [318] only was inhibited by 3 anionic polymers. Furthermore, non-enveloped adenovirus were not inhibited by any of the polymers. The attachment mechanism for non-enveloped viruses is described as a binding to cellular receptors via indentations in the capsid surface of the viruses [164,358,406]. This strengthens the interaction of the polymers with the glycoproteins of enveloped viruses.

With regards to the development of a broad-spectrum antiviral agent the *in vivo* efficiency need to be checked. Although PSVBS was able to inhibit 6 out of 11 viruses to at least 90%, it is unclear if this polymer is suitable for further investigations since it belongs to the sulfonates. Heparin, another sulfonate, is also used as anticoagulant [43,403]. PSVBS might share the same properties, so the anticoagulant efficiency also need to be clarified for this polymer. Nevertheless, PSVBS can be a candidate for the design of microbicides such as antiviral topic formulations or as lubricants for condoms to have a dual prevention of viral infections especially of sexual transmitted diseases [268,337,349] or as sprays, coatings or even paints to disinfect biomedical and biotechnological applications and to prevent any contamination with viruses [77,220].

In addition, both viruses and polymers have a negative charge. Therefore, an electrostatic repulsion between the viruses and the polymers would be expected. The interaction and the antiviral effect again emphasize the importance of the hydrophobicity of the polymers and lead to the conclusion that the amount of glycoproteins on the viral surface determines the sensitivity of the virus to antiviral polymers. Additionally, the anionic charge of the polymers increases the electrostatic repulsion of the virus from the cellular membrane, which is also negatively charged. The proposed mode of action is schematically illustrated in Figure 18.

It needs to be figured out, what exactly defines the antiviral activity in regards of the mode of action as well as the glycoprotein density. Furthermore, it is of high interest to optimize the identified lead polymer PVBzA by variation of the polymeric structure e.g. linear, branched or additional changes in the backbone of the polymer. Nevertheless, this study shows that broad spectrum antivirals are a first step towards the development of drugs that might help in future to minimize the risks of epidemic outbreaks.

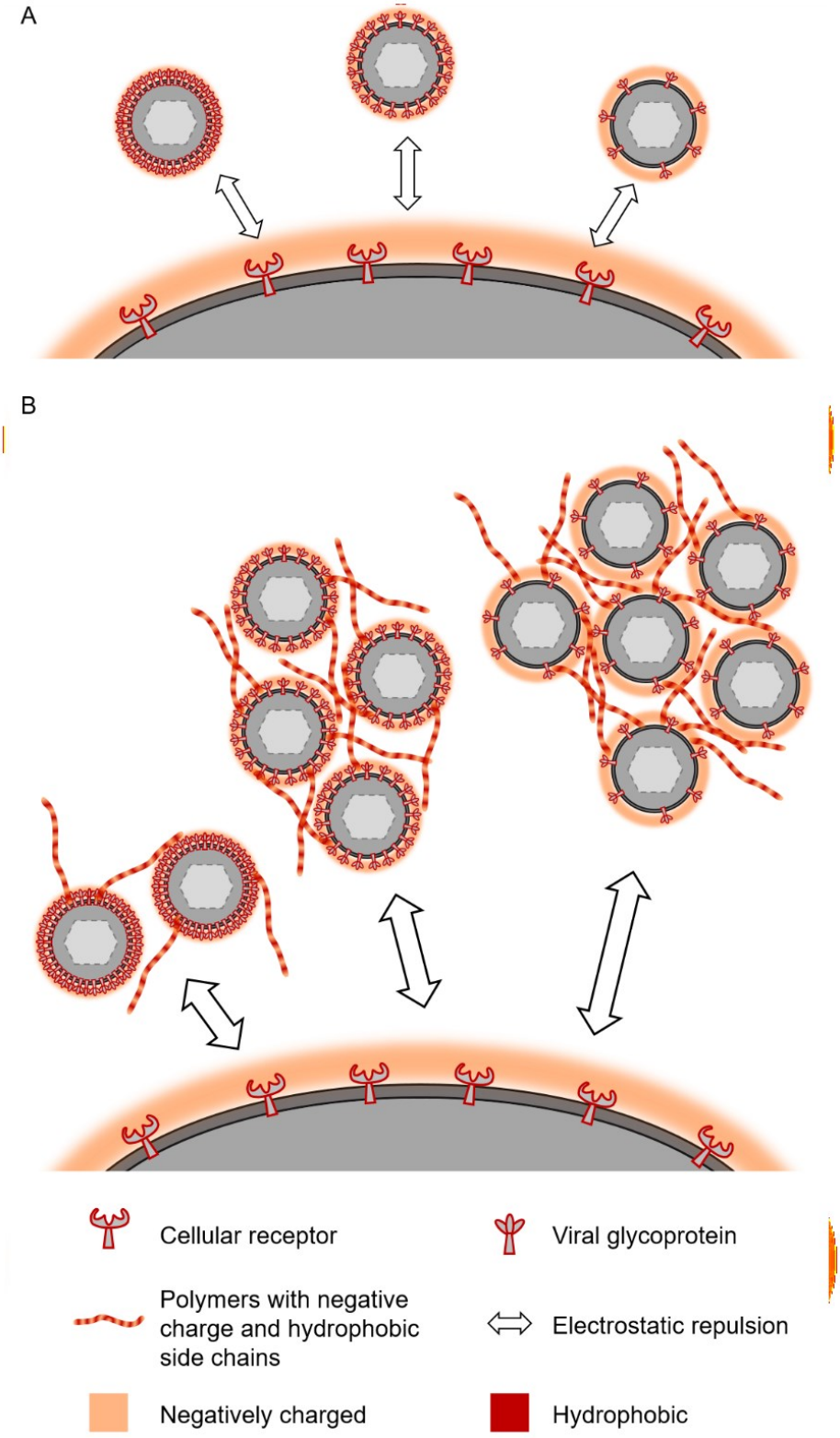

Figure 18: Mode of action for the antiviral effect of polyanions against enveloped viruses.
A) Virus-cell interaction in the absence of macromolecular agents. Enveloped virions with different amounts of viral glycoproteins bind to the cell surface via specific receptors. Due to electrostatic repulsion between the negatively charged membranes, interaction is impeded. B) Interaction of the macromolecular prodrugs with the virions. The polymers are negatively charged and have a hydrophobic character. Hydrophobicity enables the interaction with the viral glycoproteins and the viral membrane. Anionic nature increases the electrostatic repulsion thereby preventing the infection. The less glycoproteins are present on the viral surface the more polymers are able to interact with the glycoproteins.

3.3 Albumin-affine macromolecular prodrugs of acyclovir are long-acting HSV-2 inhibitors - Results

According to WHO guidelines for the treatment of herpes simplex virus infections, acyclovir is the method of choice [391]. Unfortunately, acyclovir has a low bioavailability and a short half-life [69]. To increase the pharmacokinetics of acyclovir the properties of albumin and macromolecular prodrugs were combined by designing polymers that carry acyclovir (ACV) to create albumin-affine prodrugs that inhibit HSV-2.

3.3.1 Albumin-affine macromolecular prodrugs of acyclovir that release acyclovir were synthesized

As polymer that should carry acyclovir and interact with albumin PHPMA was chosen. The drug successful drug conjugation to PHPMA has been described earlier. Those polymer-drug conjugates are water-soluble and the side-chains are stable in respect of hydrolysis, which makes PHPMA an ideal polymer candidate for drug conjugation. All compounds used in this collaborative study with Alexander N. Zelikin from Aarhus University Denmark were synthesized by him. The basis for albumin-affine drug conjugation was the synthesis of the polymer PHMPA by using the polymerizable lipid 1,2-distearoyl-sn-glycero-3-phosphoethanolamine polyethylene glycol, DSPE-PEG. The outcome of the reaction is than a polymer with affinity to albumin, called DSPE-PHPMA. Ideally, the binding results from a non-covalently interaction, which increases the flexibility of drug administration and reduces the drug handling [151,414].

The non-covalently binding affinity of DSPE-PHPMA to albumin was controlled by size exclusion chromatography with multi-angle light scattering (SEC-MALS). The higher the number of equivalent polymers attached to albumin, the shorter was the retention time (Figure 19, panel B). At a polymer to albumin ratio of 1:10 aggregates (Figure 19, A, black line) were detected, which is a result from the non-covalent albumin interaction with the polymer. Surprisingly, this is true for only 0.1% of the sample (quantified with UV-vis detector).

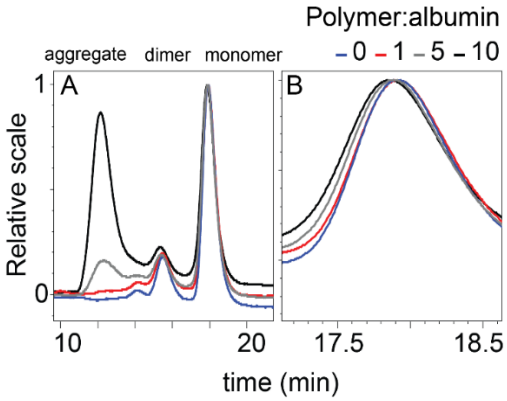

Figure 19: Interaction of DSPE-PHPMA with albumin controlled with SEC-MALS using different polymer-albumin ratios.
Size exclusion chromatography elugrams (MALS detector) illustrating the interaction between albumin and DSPE-PHPMA. Panel B is a close-up view of the albumin monomer elution region from panel A. Polymer was mixed with albumin in different ratios of 0, 1, 5 and 10 polymers per albumin. Elution of DSPE-PHPMA to albumin is documented as minutes. Experiment was performed by Camilla Kaas Frich, Department of Chemistry, Aarhus University, Denmark. Figure adapted from [114] with permission from Elsevier.

After designing albumin-affine polymers drugs, in this case acyclovir, were coupled to the polymer. The design and schematic chemical synthesis reactions leading to the albumin-affine macromolecular prodrugs are displayed in Figure 20. Shown are the single components that in the end make up the albumin-affine macromolecular prodrug, consisting of the DSPE-RAFT agent (DSPE-PEG-amine), which is used to polymerize HPMA to PHMPA, which can than carries multiple acyclovir molecules. The lipid part of the RAFT agent DSPE interacts via (non-)covalent interaction with albumin.

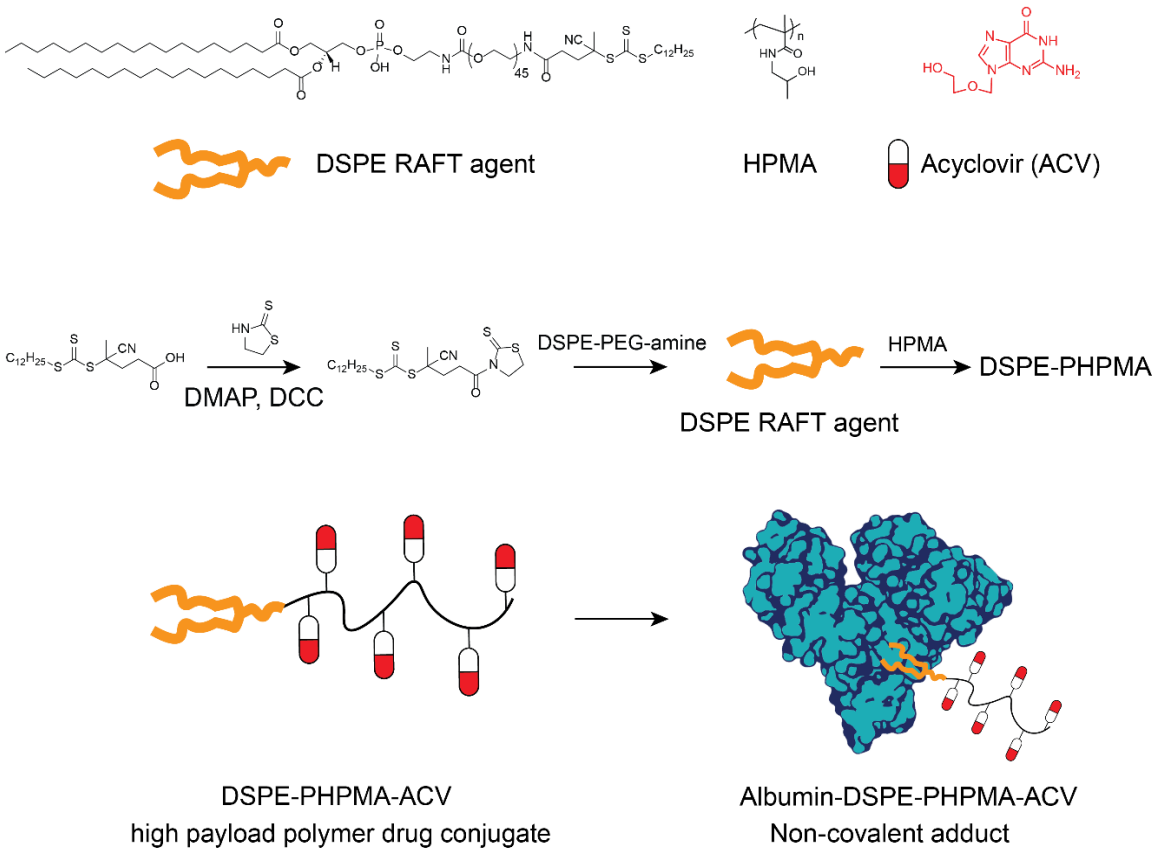

Figure 20: Schematic illustration of the generation of the Albumin-DSPE-PHPMA-ACV macromolecular prodrug. A) Structural formulas of the DSPE RAFT agent, HPMA, and acyclovir. B) Synthesis of the DSPE RAFT agent in two steps and synthesis of DSPE-PHPMA polymers. C) Schematic illustration of the DSPE-PHPMA-ACV macromolecular prodrug with affinity to albumin and the non-covalent association of MP with albumin. Figure adapted from [114] with permission from Elsevier.

By designing macromolecular prodrugs the release of the active version of the drug has to be ensured. Disulfides are suitable for this approach since they have a high stability in the blood, but react with thiols such as glutathione (GSH). GSH is a tripeptide of cysteine, glutamic acid and glycine and is abundant in mammalian cells to prevent damage by reactive oxygen species [112]. Furthermore, efficient disulfide cleavage is triggered, which can be used to release e.g. drugs from polymers [423]. In the case of albumin-affine MP this was done for acyclovir, by introducing a so called self-immolative linker composed of a disulfide [280] that undergoes disulfide-reshuffling to a thiol in the presence of GSH [203]. In the end, this leads to the release of the active drug (schematic illustration in Figure 21, A).

The release of acyclovir from the macromolecular prodrugs, PHPMA-ACV, was quantified using HPLC (done by Raoul Walther, Department of Chemistry Aarhus University, Denmark). In agreement with the design considerations, MPs release insignificant quantities of acyclovir in physiological buffer (phosphate buffered saline) even over 24 h of incubation (Figure 21, B). In contrast, in the presence of GSH taken at a physiologically relevant concentration of 5 mM, the polymer readily released significantly acyclovir (Figure 21, A, B). This validates the proposed design of the monomer acyclovir equipped with a self-immolative linker.

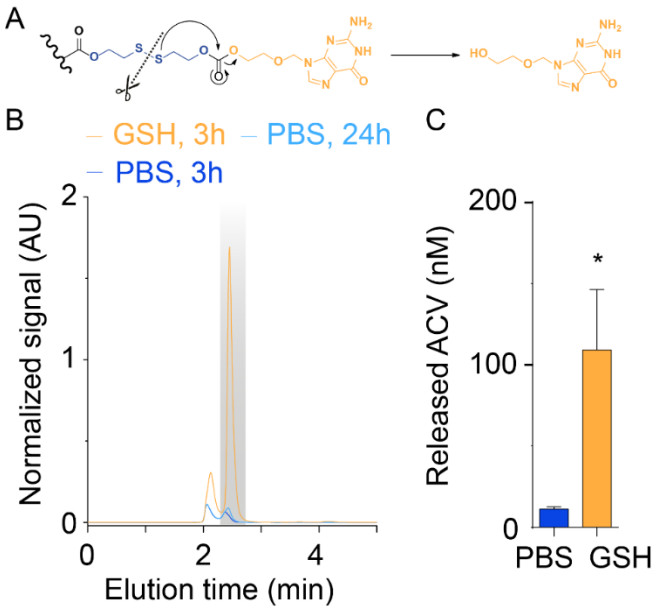

Figure 21: Schematic illustration of the release of ACV from the macromolecular prodrug after disulfide reshuffling.
A) Illustration of the mechanism of drug release for ACV prodrugs upon disulfide reshuffling. B) Macromolecular prodrug (MP) of ACV was incubated at 37°C for 3 h with 5 mM glutathione (GSH). The polymer was removed via spin filtration and filtration was analyzed with HPLC. HPLC traces depicting the release of ACV from its MP in the presence of 5 mM GSH in PBS (10 mM, pH = 7.4) after 3 h or PBS alone (3 and 24 h). C) Amount of released ACV from the MP in presence of GSH or PBS. Quantitative data is represented as mean ± standard deviation from 3 independent experiments. * $p < 0.01$. Experiment was perforemed by Raoul Walther, Department of Chemistry, Aarhus University, Denmark. Figure adapted from [114] with permission from Elsevier.

3.3.2 Albumin-affine macromolecular prodrugs of acyclovir inhibit HSV-2 infection *in vitro*

Next, the following macromolecular prodrugs of acyclovir were generated and evaluated for their antiviral activity (Table 4). For this, the genetically engineered baby hamster kidney reporter cell line ELVIS™ was incubated with the different compounds prior infection with HSV-2. Infection was determined after two replication rounds of HSV-2 by measuring the β-galactosidase activity of the reporter cell line. To exclude cytotoxic effects of the macromolecular prodrugs ELVIS™ cells were further incubated with the compounds without infecting the cells. Metabolic activity was determined an MTT assay.

Table 4: Molecules used to investigate the antiviral activity of albumin-affine macromolecular prodrugs.
As not indicated otherwise the RAFT agent to synthesize the polymer PHPMA was DSPE-PEG. The acyclovir (ACV) content varied between 6.5 and 12%. DSPE non-covalently interacts with albumin. To study the difference in efficiency the polymer as well as the macromolecular prodrug were covalently linked to albumin. Table adapted from [114] with permission from Elsevier.

Designation	Specific properties
PHPMA (P1)	RAFT agent: OH
PHPMA-ACV (P2)	RAFT agent: OH; ACV content of 6.5 mol %
DSPE-PHPMA-ACV (P7)	ACV content of 7 mol %
DSPE-PHPMA-ACV (P8)	ACV content of 12 mol %
Albumin-co-PHPMA	PHPMA covalently linked to albumin
Albumin-co-(PHPMA-ACV)	PHMPA-ACV covalently linked to albumin; ACV content of 7 mol %

Acyclovir and heparin, which is described as inhibitor of HSV-2 entry [357] were used as controls. The polymer PHPMA showed a reduced infection at the highest concentration (Figure 22, red line in A). For the MPs of acyclovir a dose-dependent inhibition to remaining 40% (Figure 22, red line in B-D and F) was achieved. The same was observed for acyclovir (Figure 22, red line in H). Only heparin, the viral entry inhibitor, inhibited the infection completely (Figure 22, red line in G). A possible explanation for the incomplete inhibition of the MPs is the temporal difference of the β-galactosidase expression of the used cell model to the viral thymidine kinase expression, necessary for phosphorylation of acyclovir and the resulting incorporation into the viral DNA [98]. The β-galactosidase expression is detectable already after 6 h [334], whereas the expression of thymidine kinase is a late event in the replication cycle of HSV-2 [205,366,378]. This results in a plateau of β-galactosidase activity even at the highest concentration of the macromolecular prodrugs (Figure 22, red lines in B-D, F and H). Compared to the concentrations that were required for antiviral activity of the native acyclovir, the concentrations for the macromolecular prodrugs were much lower (maximum 100 µg/ml for MPs and 1000 µM for acyclovir). By comparing the cells when treated with the different compounds and controls none or only slightly impaired the cell viability (Figure 22, black lines).

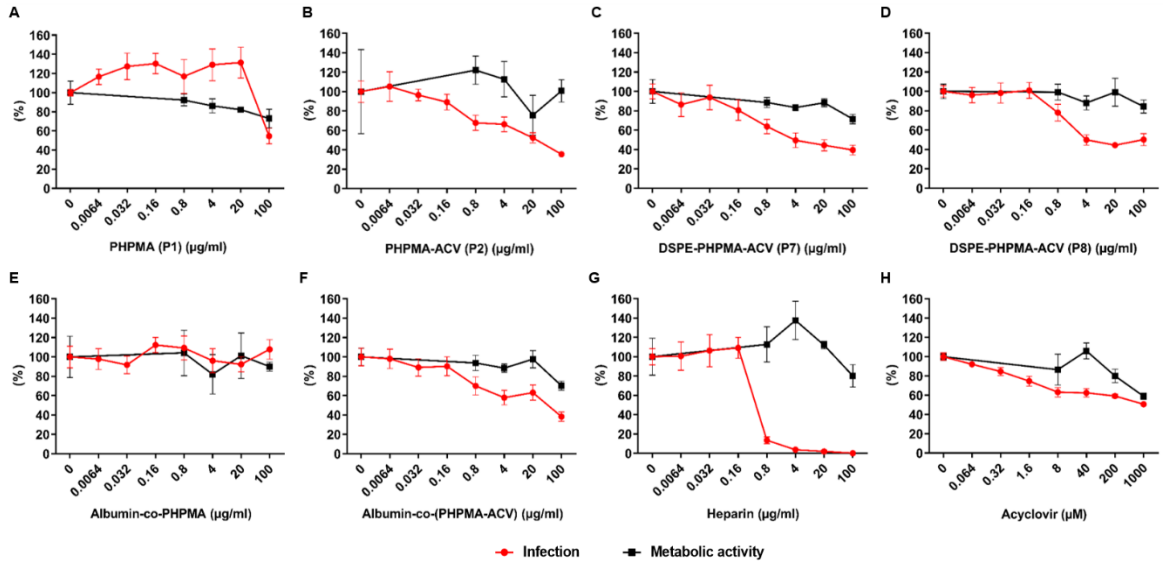

Figure 22: Dose response and metabolic activity curves of ELVIS^{TM} cells reflecting activity of PHPMA, MPs of ACV, ACVand heparin in suppressing HSV-2 replication *in vitro*.
ELVIS^{TM} cells were treated with A) PHPMA, B-F) MPs of ACV as well as G) heparin and H) ACV 1 h prior infection with HSV-2. Infection (red lines) was determined 48 h post infection by quantifying β-galactosidase activity in whole cellular lysates. Data are normalized to infection in the absence of the respective polymer. Metabolic activity (black lines) was determined by MTT assay of ELVIS^{TM} cells incubated with A) PHPMA, B-F) MPs of ACV as well as G) heparin and H) ACV for 48 h in absence of virus. Data are normalized to metabolic activity of cells in the absence of the respective polymer. Values shown are means ± SEM derived from three independent experiments performed in triplicates. Figure adapted from [114] with permission from Elsevier.

Due to the interference of the β-galactosidase signal with the expression of the thymidine kinase HeLa cells were used to investigate the antiviral effect of the macromolecular prodrugs in this cell line. Since HeLa cells do not show cytopathic effects upon infection with HSV-2 [46] I used an HSV-2 eGFP and fluorescence microcopy to visualize the

antiviral activity. Furthermore, the eGFP expression does not interfere with the expression of the thymidine kinase [330]. Again, HeLa cells were incubated with the previously tested compounds prior infection. After 3 replication rounds the cells were fixed, stained for nuclei and fluorescence microscopy was performed (Figure 22). The acyclovir macromolecular prodrugs PHPMA-ACV, DSPE-PHPMA-ACV (P7 and P8) and albumin-co-(PHPMA-ACV) decreased infection almost entirely at 100 µg/ml, (Figure 23). In contrast, PHPMA alone or the carrier covalently coupled to albumin (Figure 23) were not able to inhibit HSV-2 infection. The controls heparin and acyclovir completely inhibited the infection already at the lowest concentration 0.8 µg/ml respectively 8 µM.

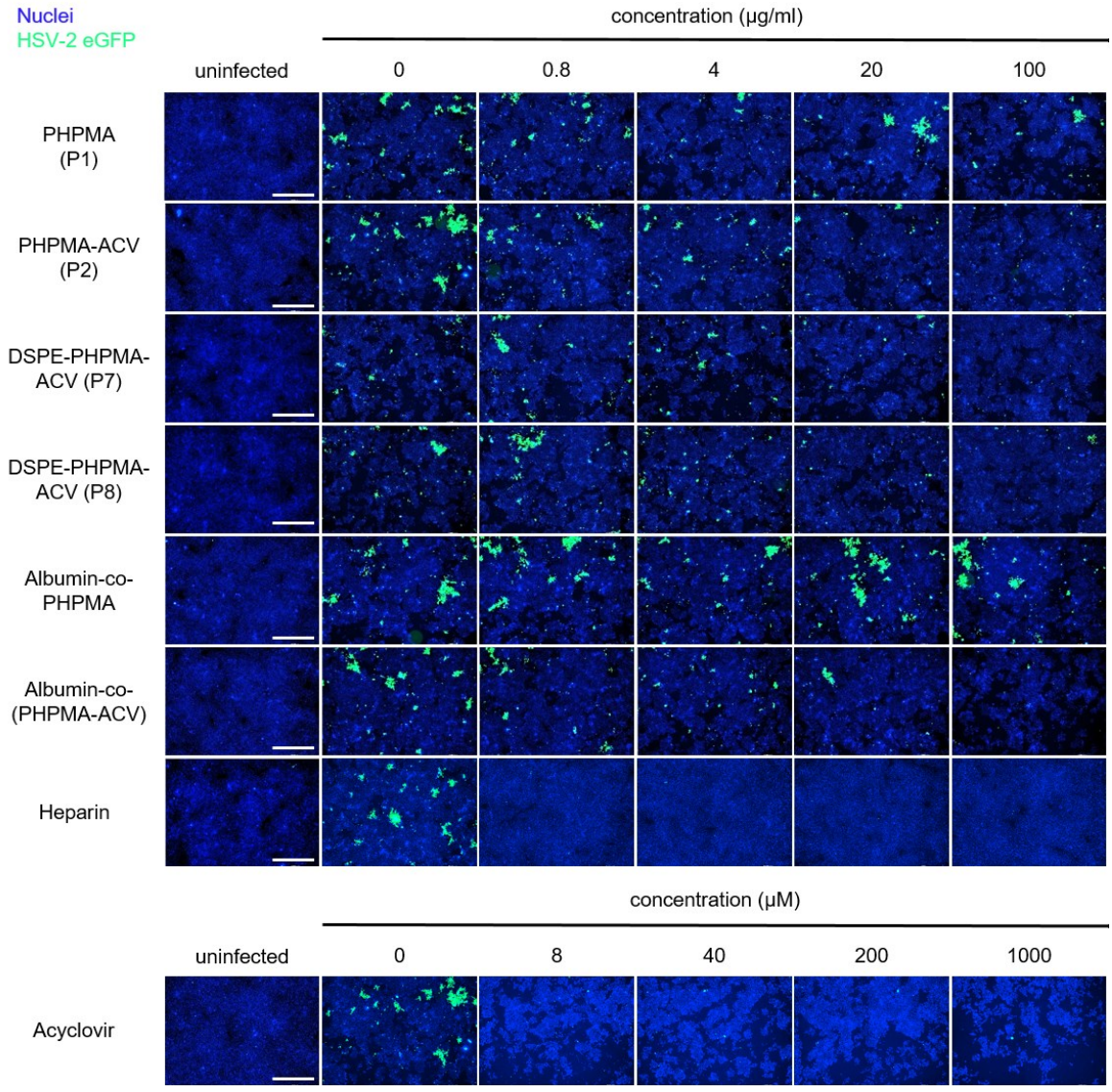

Figure 23: Fluorescence microscopy images of HeLa cells infected with HSV-2 reflecting activity of PHPMA, MPs of ACV, ACVand heparin in suppressing HSV-2 replication *in vitro*.
HeLa cells were incubated with the compounds for 1 h prior infection with HSV-2 eGFP. 96 h post infection, cells were fixed and stained for nuclei (blue). Microscopy image acquisition was conducted. Experiment was performed in triplicates, one representative image is shown for each condition. Scale bar is 1000 µm (same for all panels). Figure adapted from [114] with permission from Elsevier.

Furthermore, for the exclusion of any cytotoxic effects HeLa cells were incubated with the compounds without infecting them. After 3 days an MTT assay was performed which is a method to determine the metabolic activity of the cells as sign for the cell viability. Only acyclovir had a strong impact on the metabolic activity at the highest concentration of 1000 µM (Figure 24, B), which is much higher as the concentrations that were used to conjugate acyclovir to the macromolecular prodrugs. Hence, all other tested compounds did not affect the metabolic activity. (Figure 24, A). Surprisingly, DSPE-PHPMA-ACV (P8) and Albumin-co-(PHPMA-ACV) increased the metabolic activity.

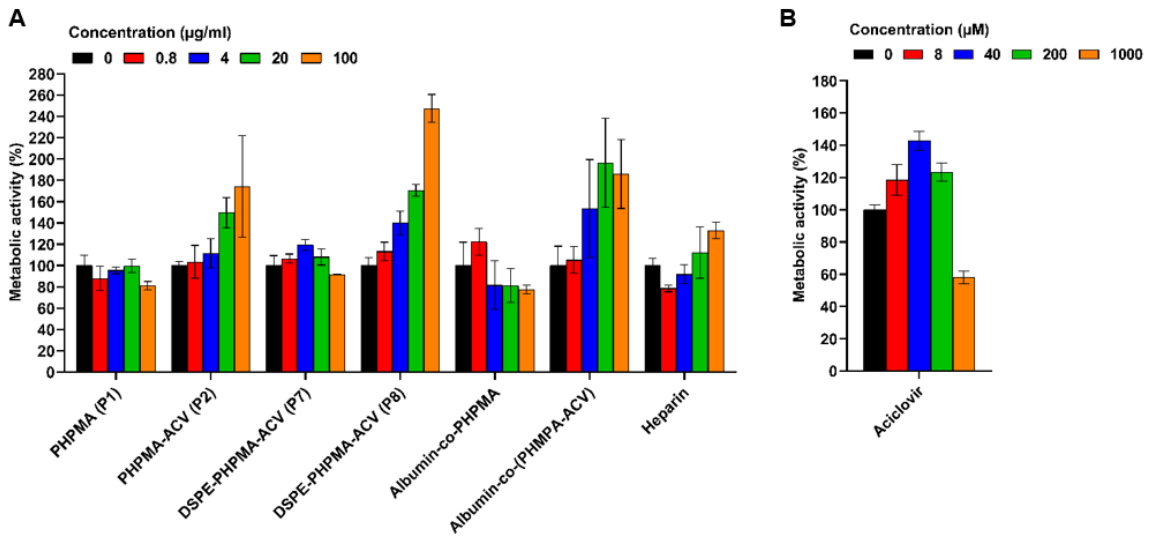

Figure 24: Metabolic activity of HeLa cells cultured in the presence of PHPMA, MPs of ACV, ACV and heparin at concentrations used in respective antiviral screens.
HeLa cells were incubated with A) PHPMA, MPs of ACV and heparin as well as B) with acyclovir. After 96 h MTT assay was performed to determine the metabolic activity of the cells. Data are normalized to the metabolic activity of cells in absence of the compounds. Shown are mean values derived from biological triplicates ± standard error.

3.3.3 High blood and body residence time of albumin-affine macromolecular prodrugs in mice

Macromolecular prodrugs should increase the bioavailability and body residence time by non-covalently binding to albumin, which is present in the blood and extravascular tissue [90]. To experimentally test this, MPs were administered via subcutaneous (s.c.) and intravenous (i.v.) injection. Specifically, DSPE-PHPMA, albumin-co-PHPMA and PHPMA were labelled with the fluorophore Cy7 was injected in female Balb/C mice s.c. in the scruff of the neck or i.v. in the tail vein (Paul W. Denton and Martin Tolstrup, Aarhus University, Denmark). Up to 7 days post injection blood samples were drawn and centrifuged to separate plasma and cells. Images were acquired and fraction of compound in the plasma was estimated on the measured fluorescence intensity. The mean amount of each compound in plasma is related to its maximum fluorescence of the specified injection route. Although the absolute concentration of polymer in the blood expressed as fluorescence signal was higher after i.v. administration (Figure 25, A; log 7.5 AU for s.c. vs. log AU for i.v.), DSPE-PHPMA was longer detectable after s.c. administration (Figure 25, B). Furthermore, the maximum concentration of DSPE-PHPMA after i.v. administration was instantly reached.

For the s.c. administration this was the case after 7 h. After 24 h DSPE-PHPMA administered i.v. was not detectable anymore with a half-life of 138 min, whereas at the subcutaneous administered DSPE-PHPMA was still detected after 3 days with a half-life of around 18 h (Figure 25, B). For long-acting drugs this is a highly favored property. By comparing the total amount of DSPE-PHPMA with PHPMA covalently coupled albumin and PHPMA for 7 days it is observed that PHPMA covalently and non-covalently coupled to albumin share similar drug decreasing profiles (Figure 25, C). Furthermore, between 2 and 12 h post administration a plateau of DSPE-PHPMA and PHPMA covalently coupled to albumin is detectable between 80 and 100% of the maximum measured drug amount in the blood. This is not the case for PHPMA alone which already reached the maximum concentration after 4 h, but DSPE-PHPMA and albumin-co-PHPMA only after 6 h (Figure 25, C). The difference in the distribution profile between PHPMA and DSPE-PHPMA/albumin-co-PHPMA was significant at 6 h ($p<0.05$) and 12 h ($p<0.01$). Even after 24 and 48 h the concentration of PHPMA covalently or non-covalently coupled to albumin. is 30%, respectively 20% of the maximum dose that was observed in the blood after s.c. administration (Figure 25, C). This leads to the assumption that albumin is able to extent the circulation time of the drug PHPMA in the body after s.c. administration route, regardless if PHPMA was covalently or non-covalently coupled to albumin.

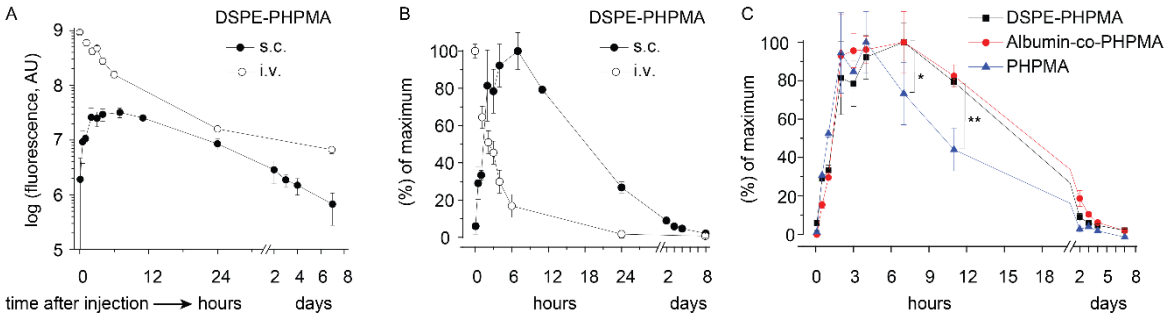

Figure 25: Quantification of PHPMA, DSPE-PHPMA, and covalent conjugate between PHPMA and albumin in mice blood following intravenous or subcutaneous administration over 7 days.
A) Quantification of DSPE-PHPMA in blood. B) Concentration of DSPE-PHPMA in blood normalized to the highest value observed for the respective route of administration; C) Quantification of the solutes in blood upon s.c. administration. Fractions were compared with an unpaired t-test; * represents $p<0.05$ and ** is $p<0.01$ For all graphs, each data point represents an average of 4 individual mice ± SEM. Data for PHPMA and Albumin-co-PHMPA are taken from (ref). Experiments were performed by Anna Folkmar Halling Andersen, Department of Infectious Diseases/Department of Clinical Medicine, Aarhus University Hospital and Department of Clinical Medicine, Aarhus University. Figure adapted from [114] with permission from Elsevier.

To identify how PHPMA, DSPE-PHPMA (i.v. and s.c.) and albumin-co-PHPMA is distributed into different organs the amount of the fluorescent labelled compounds has been analyzed in lymph nodes, liver spleen and lungs. Therefore, mice exposed to the different compounds terminated under anesthesia at 1 h, 24 h and 7 days. Blood was displaced from the organs by PBS perfusion through the heart. After removing the organs they have been scanned with an *in vivo* imaging system. To investigate an accumulation or depletion of the administered PHPMA, DSPE-PHPMA and albumin-co-PHPMA the amount of the compounds measured in different organs after 24 h and 7 days (Figure 26) was normalized to the amount measured after 1 h. For all tested compounds the amount was reduced after 7 days compared to 24 h, except for the lymph nodes in which the values remained constant

or even increased after 7 days. After i.v. administration, DSPE-PHPMA was nearly completely eliminated from all organs. This was not the case for DSPE-PHPMA or PHMPA when administered s.c. The highest organ accumulation was observed when covalently coupled PHPMA to albumin was administered s.c. The difference in the organ accumulation profiles (Figure 26) combined with the results from biodistribution in the blood (Figure 25) highlight the assumption that for non-covalently DPSE-PHPMA albumin is responsible for the transition of DSPE-PHPMA from the subcutaneous space to the blood, whereby PHPMA contributes to the body and organ distribution.

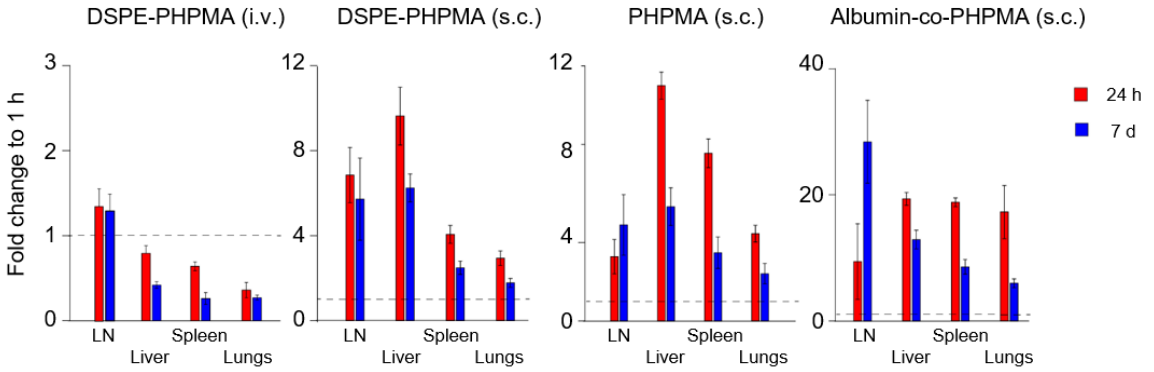

Figure 26: Biodistribution of subcutaneously administered albumin-affine macromolecular prodrugs after 24 h and 7 d in different organs.
Quantification of DSPE-PHPMA (i.v. and s.c.), PHPMA (s.c.) and PHPMA covalently coupled to albumin (s.c.) rates in mice organs after mice were euthanized and blood was eliminated from organs with PBS perfusion through the heart. Image acquisition of the single organs was performed with in vivo imaging. Lymph nodes (LN), liver, spleen and lungs. Samples were taken at 1 h, 24 h and 7 days. Values are shown as fold-change compared to the 1 h timepoint set to 1. Experiments were performed by Anna Folkmar Halling Andersen, Department of Infectious Diseases/Department of Clinical Medicine, Aarhus University Hospital and Department of Clinical Medicine, Aarhus University. Figure adapted from [114] with permission from Elsevier.

3.3.4 Albumin-affine macromolecular prodrugs of acyclovir prevent HSV-2 infection *in vivo*

To investigate the antiviral potential of albumin-affine macromolecular prodrugs the prevention of HSV-2 infection has been investigated *in vivo* (Søren R. Paludan, Aarhus University, Denmark).

PHPMA-ACV, DSPE-PHPMA-ACV or acyclovir were injected s.c. at a dose matching 60 mg/kg acyclovir dosing, which is a standard orally administered dose for this model. 5 h later 5 mice of each treatment group as well as a non-treated group were infected vaginally with HSV-2. Mice were observed for 7 days and each day the clinical score was determined (Figure 27, A). Mice with a clinical score over 3 (purulent lesions, partial or complete paralysis in one or both limbs, weight loss or dehydration [223]) were sacrificed. Since HSV-2 not only affects the perianal and genital tract of mice, but also the central nervous system [381] the virus titer at day 2 and day 7 was not only quantified in vaginal lavage and medulla spinalis (Figure 27, B).The highest clinical scoring was observed already 5 days after infection of mice which did not receive any treatment. In mice treated with acyclovir no clinical symptoms of infection were observed and the viral titers in the vaginal wash as well

as in medulla spinalis remained below the detection limit (Figure 27, A). In the group treated with PHPMA-ACV as well as in the group treated with DSPE-PHPMA-ACV only one out of five mice showed symptoms of an HSV-2 infection and a mild disease progression. The clinical scoring remained below 1, but viral loads were above the detection limit in vaginal wash as well as in medulla spinalis. In comparison, the untreated control showed a significant disease progression and high viral loads (Figure 27). All other mice were comparable to the mice treated with acyclovir, which neither developed clinical symptoms nor exhibited detectable virus loads in vaginal lavage and medulla spinalis. Macromolecular prodrugs of acyclovir prevent infection and disease progression of HSV-2 *in vivo*.

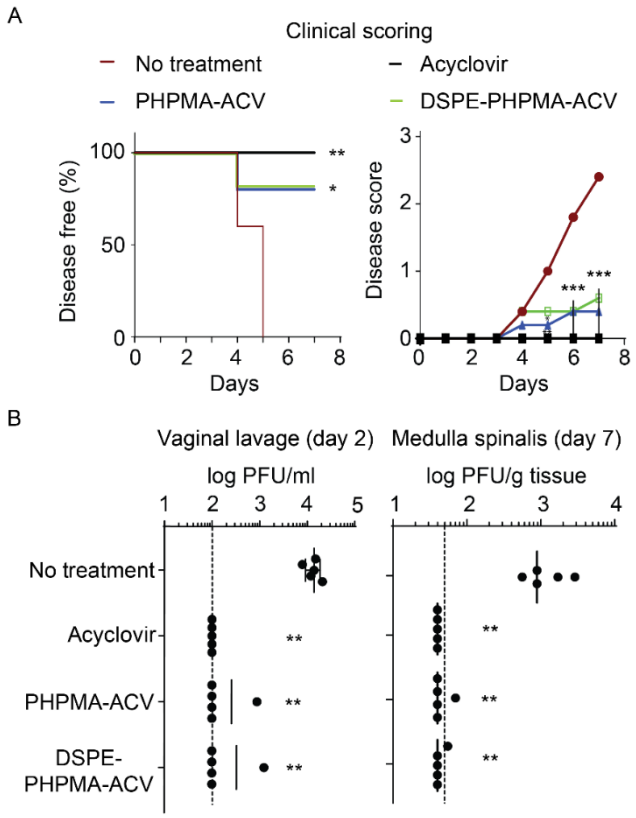

Figure 27: Analysis of HSV-2 infection in mice treated with macromolecular prodrugs of acyclovir.
Mice were infected with HSV-2. 5 h post infection they were treated with the respective compounds acyclovir, PHPMA-ACV and DSPE-PHPMA-ACV all taken at 60 mg/kg equivalent dose of ACV. A) Kaplan-Meier plot (disease free animals (%), treatments were compared to non-treated using a log-rank (Mantel-Cox) test and average disease scores. 0=healthy; 1=genital erythema; 2:moderate genital inflammation; 3=purulent genital lesion/generally bad condition; 4=hind limb paralysis/generally poor condition. Mice were sacrificed by cervical dislocation at score exceeding 3. Means were compared to non-treated by a two-way ANOVA at each time point. B) Virus titer in vaginal lavage obtained at day 2 and medulla spinalis at day 7 post infection, determined with virus plaque assay to assess the amount of plaque forming units (PFU). Treatments were compared to the non-treated group using the Kruskal-Wallis tests with Dunn's multiple comparisons tests. Dashed lines indicate the detection limit. * $p< 0.05$, ** $p<0.01$, *** $p<0.001$. Experiments were done done by the group of Søren R. Paludan, Department of Biomedicine, Aarhus University. Figure adapted from [114] with permission from Elsevier.

3.4 Macromolecular prodrugs with albumin affinity as HSV-2 inhibitors - Discussion

Many people suffer from Herpes simplex virus type 1 and 2 infections. Nearly 4,200 million people were infected in 2012 [214,215]. To treat the infections the nucleoside analogue acyclovir is recommended [391]. To increase its bioavailability PHPMA, has been synthesized to which high amounts of acyclovir were coupled by a self-immolative linker [280]. This results in the release of the parent drug by disulfide-reshuffling in mammalian cells mediated by glutathione [203]. Indeed, PHPMA-ACV was superior in inhibiting HSV-2 infections *in vitro* compared to acyclovir itself, which was also toxic at the highest concentrations used. Furthermore, to realize an albumin-affine macromolecular prodrug that extend the body half-life of PHPMA-ACV, the lipid DSPE was used, which interacts with albumin via a non-covalently interaction [209]. In *in vitro* assays no differences in the antiviral potential between DSPE-PHPMA-ACV and the covalently coupled albumin-co-PHPMA was observed, highlighting that the development of the macromolecular prodrugs were successful. The proposed drug circulation, the release of the active monomer and the antiviral mode of action are depicted in Figure 28.

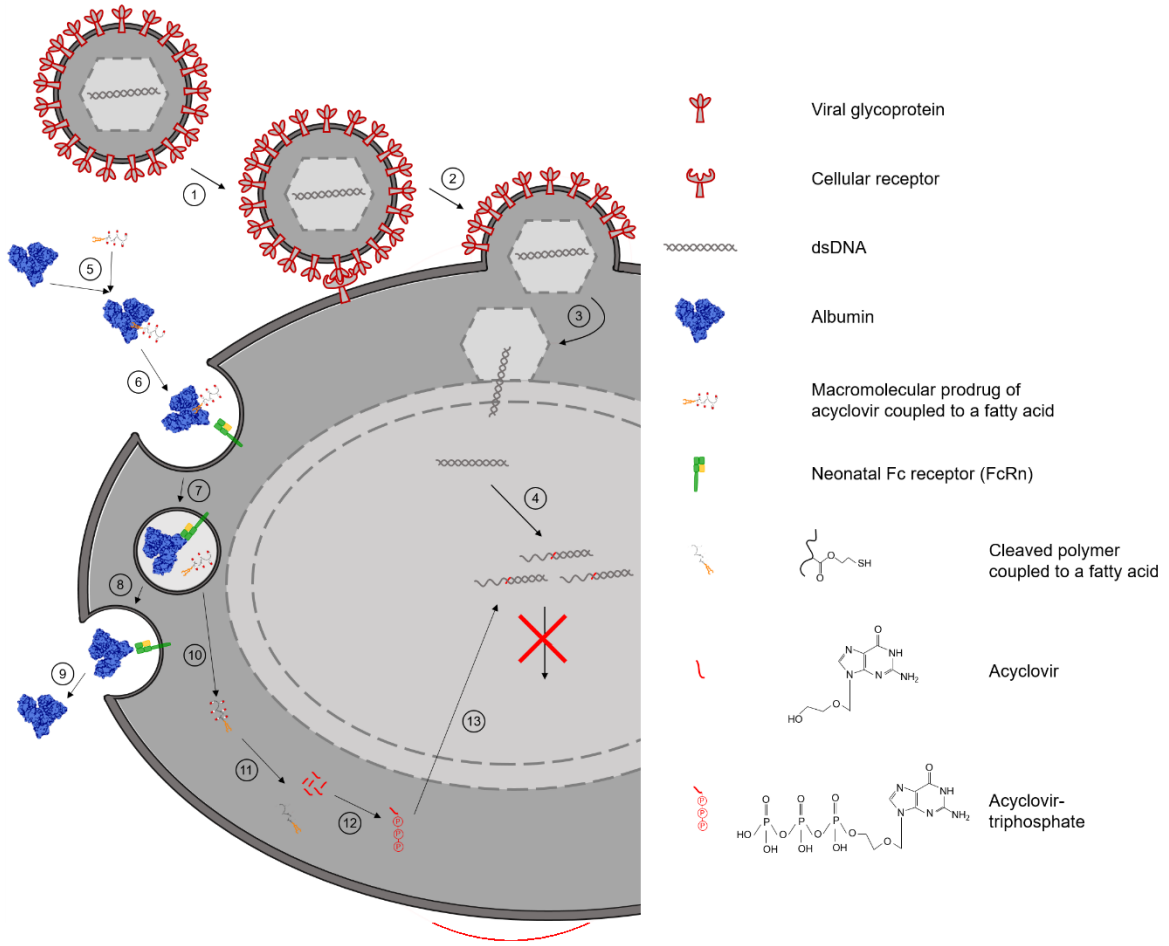

Figure 28: Schematic illustration of the mode of action of albumine-affine macromolecular prodrugs.
1) HSV attaches to the cell surface via interaction of viral glycoportein with cellular receptors. 2) Viral envelope fuses with the plasma membrane. The capsid is released into the cytoplasm and capsid is transported to the nuclear pore. 4) Viral DNA is released into the nucleus and replication of the DNA starts. 5) Non-covalent interaction of the macromolecular prodrugs of acyclovir coupled to a fatty acid with albumin. 6) Non-covalently interacting macromolecular prodrug of acyclovir with albumin is uptaken by the cell. 7) Endosomes are formed and albumin binds to the FcRn while the macromolecular prodrug gets detached from the albumin. 8) Albumin is transported to the cell membrane and 9) is released under physiological pH. 10) The macromolecular prodrug of acyclovir coupled to a fatty acid is processed further. 11) Desulfide reshuffling by glutathione leads to the release of acyclovir from the polymer. 12) Three phoyphorylation steps lead to the activation of acyclovir which is transported into the nucleus and incorporated into the replicating viral DNA. This leads to the strand termination and the inhibition of HSV.

In vivo experiments in mice were performed to determine the most suitable administration route of albumin-affine macromolecular drugs. After intravenously administration the levels of the prodrugs dropped much faster compared to the subcutaneous administration of the formulation. Furthermore, the absolute blood concentration of the albumin-affine polymer was much higher after intravenous administration [5,274], whereas the concentrations were more stable over 7 days after subcutaneous administration. For the development of clinical useful drug formulations these results are favored, since it argues for a long bioavailability of the drug.

Since the administration profiles of PHPMA, which was covalently or non-covalently (via DSPE) coupled to albumin, are almost identical, a non-covalently coupling is preferred with regards to further drug development with prolonged half-life mediated by albumin. Non-covalently formulations benefit from their ease of handling as well as flexibility of drug

administration in comparison to covalently coupling to albumin [185,197]. Furthermore, the organ accumulation of albumin-affine macromolecular prodrugs after intravenous and subcutaneous administration showed a nearly complete elimination of DSPE-PHPMA from all organs, excluding the lymph nodes. This possibly will be an interesting approach for vaccination, whereby PHPMA can help to improve the antigen delivery to lymphoid organs for initiating the immune response [163,238]. Due to the high accumulation in the lymph nodes PHPMA can also act as an adjuvant with depot-forming potential [138].

For clinical applications of polymers their molar mass is crucial for its properties, as it determines the hydrodynamic radius and therefore the blood circulation time [343]. With the synthesis of the albumin-affine prodrugs this was avoided. Blood circulation and organ accumulation of the prodrugs showed that the pharmacokinetic characteristics of acyclovir are transferred to albumin (blood circulation) and the polymer (organ distribution). This is a useful strategy for creating long-acting drugs [5,423].

In vivo analysis revealed that the disease progression of mice infected with HSV-2 was significantly slowed down by the macromolecular acyclovir prodrugs. In each group treated with PHPMA-ACV or DSPE-PHPMA-ACV, one out of five mice showed disease progression accompanied by virus titers above the detection limit. In the mice treated with acyclovir neither symptoms nor detectable virus loads were observed. Although the disease was not prevented in all mice when treated with DSPE-PHPMA-ACV, it shows that the drug formulation can be considered for further *in vivo* analysis. The question remains, why acyclovir was still superior to the treatment with DSPE-PHPMA-ACV, since all mice had virus loads below the detection limit when treated with acyclovir. Furthermore, the intravenous administration of DSPE-PHPMA-ACV should be studied. Although the results with the subcutaneous administration are highly encouraging, the concentrations after i.v. were still higher. With regards to a fast mode of action i.v. administration was superior to a subcutaneous administration. Furthermore, trying to increase the payload of acyclovir coupled on the carrier PHPMA might also result in a better outcome of mice infected with HSV-2. Since the mice were treated shortly after infection the treatment is much more a preventive event. Of high interest is also a situation where the infection is already present to see if the symptoms can be reduced after a treatment with DSPE-PHPMA-ACV. Nevertheless, macromolecular prodrugs of acyclovir were efficacious *in vitro* and *in vivo*. Subcutaneous administration was able to prevent disease progression in mice, highlighting that this formulation can be used for systemic antiviral treatment. In the case of acyclovir, resistant strains can still not be targeted. Therefore, further drugs that counteract the viral resistant strains need to be developed. There are already drugs on the market that circumvent the phosphorylation step by the thymidine kinase e.g. foscarnet [381]. Foscarnet also shows low bioavailability [265], but possibly will benefit from a non-covalently coupling to albumin via a prodrug formulation like DSPE-PEG to treat acyclovir-resistant strains.

The design of such macromolecular prodrugs opens a lot of opportunities in creating drugs to increase the bioavailability [104], to increase the drug payload [134], to increase the solubility of a drug [183,297,359], to increase the stability regarding blood and tissue residence time [345] and to formulate systemic applications [197].

3.5 Therapeutic Nucleic Acids of the nucleoside analogue idoxuridine inhibit HSV infection - Results

The first antiviral drug on the market was Idoxuridine and was used for the treatment of HSV infections [167,270]. Another drug to treat HSV infections is Trifluridine [64,271]. Both drugs are nucleoside analogues of thymidine (Figure 29) and they inhibit viral replication by being falsely incorporated into the viral DNA leading to a strand termination [67,363]. Unfortunately, both Idoxuridine and Trifluridine have a low bioavailability [362] and are not very specific regarding their target. They also can be incorporated during the replication cycle of non-infected cells which in the end leads to adverse effects [381]. Therefore, the use of them as antiviral agents to treat HSV induced keratoconjunctivitis has been discontinued. To optimize this drug a polymer is preferred which is possibly taken up by the cell and which releases the active monomeric version thereby blocking the viral replication.

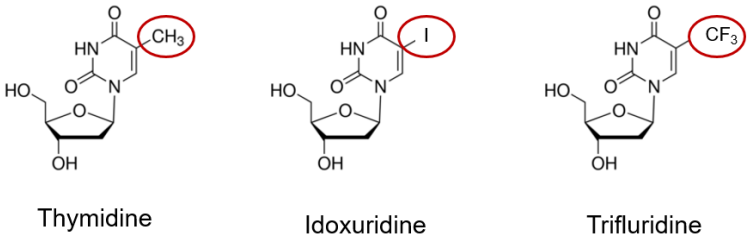

Figure 29: Chemical formulas of thymidine and the respective nucleoside analogues Idoxuridine and Trifluridine. The structural differences are highlighted in the red circle.

3.5.1 Therapeutic Nucleic Acids are the first generation of nucleoside analogue polymers are fragmented by nucleases into the monomeric nucleosides

To synthesize nucleic acids with different compositions thymidine and the nucleoside analogues Idoxuridine and Trifluridine have been used (Table 5). The nucleic acids containing thymidine should provide information about the structure-activity relationship of the therapeutic nucleic acids.

Table 5: Therapeutic nucleic acids (TNAs) and their composition

Name	Therapeutic nucleic acid composition
TNA-I_{14}	14 monomers of Idoxuridine
TNA-F_{14}	14 monomers of Trifluridine
TNA-T_{14}	14 monomers of thymidine
TNA-I_7	7 monomers of Idoxuridine
TNA-F_7	7 monomers of Trifluridine
TNA-T_7	7 monomers of thymidine
TNA-T/I_7	Alternating sequence of 7 monomers of thymidine and Idoxuridine each
TNA-T/F_7	Alternating sequence of 7 monomers of thymidine and Trifluridine each

The polymerization reaction forming therapeutic nucleic acids out of nucleoside monomers (Figure 30, A) is shown in in Figure 30, B. 7-mers as well as 14-mers were the result of the synthesis and purity was confirmed by using mass-spectrometry, HPLC and MALDI (Figure 30, C-E), where a specific peak for each TNA is visible. By injecting TNA-I_{14} and TNA-I_7 together, single defined peak with high intensity for each polymer (Figure 30 D, E) was observed, concluding that the macromolecular prodrugs are monodisperse. Compared to other polymerization techniques such as RAFT, TNA synthesis is superior regarding the composition and homogenicity of the prodruct [41,236,423].

To test the nucleolytic fragmentation of the TNAs they were incubated with a nuclease/phosphodiesterase, and analyzed by LC-MS (Figure 30, F). Treatment with the nuclease/phosphodiesterase revealed a complete degradation of TNAs and release of the expected nucleosides thymidine, trifluridine or idoxuridine, confirming the correct composition of the TNAs Alexander N. Zelikin, Aarhus University, Denmark).

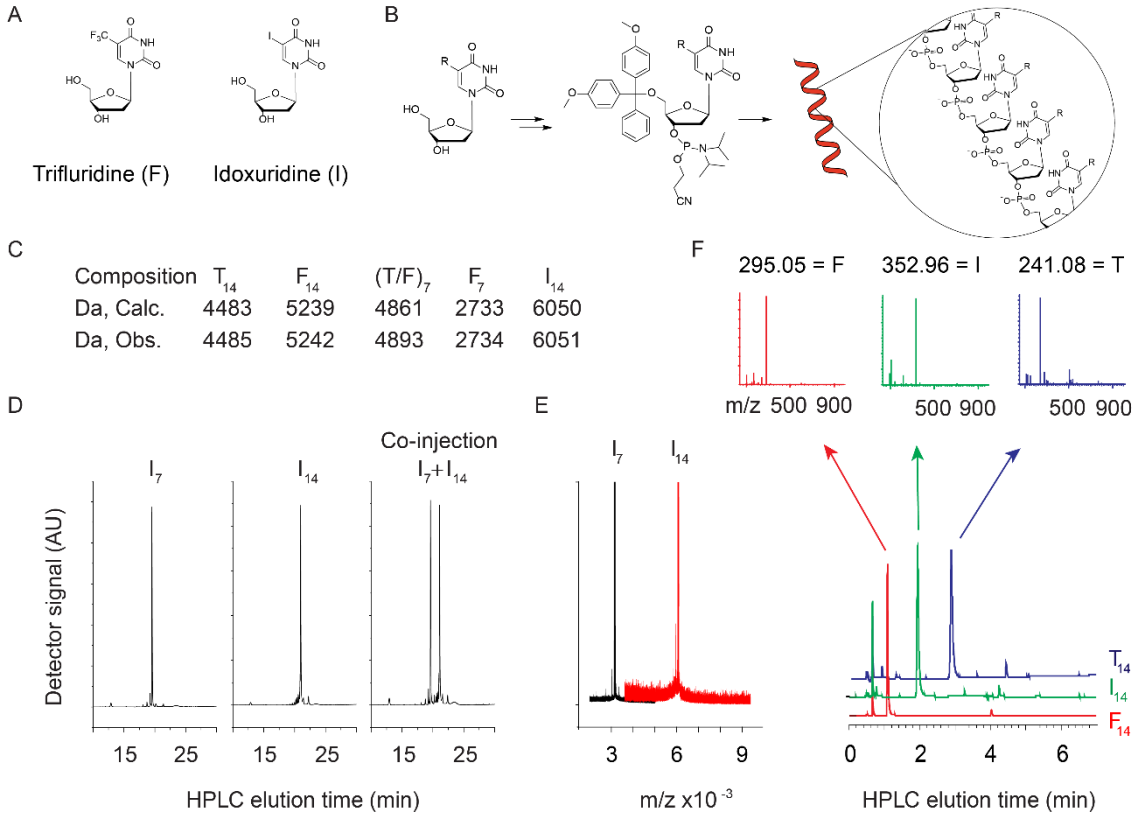

Figure 30: Overview of the composition of therapeutic nucleic acids and their degradation by nucleases.
A) Chemical formulas of trifluridine and idoxuridine. B) Schematic illustration for the conversion of antiviral drugs into monomers suitable for automated synthesis of nucleic acid (R=I for idoxuridine and R=CF3 for trifluridine). C) Mass spectrometry characterization of TNA with regards to the molar mass. D) HPLC characterization of TNA-I_7 and TNA-I_{14}. Sequence elute at distinctly different times, as individual peaks, with minimal content of shorter oligomers thus illustrating that TNA are essentially mono-disperse reaction products. E) MALDI characterization of the TNA-I_7 and TNA-I_{14} supporting the notion that TNA are obtained as mono-disperse reaction products. F) LC-MS analyses illustrate that upon exhaustive digestion (phosphodiesterase I from *Crotalus adamanteus* venom), TNA release the expected idoxuridine, trifluridine or thymidine. Figure adapted from [186].

3.5.2 Therapeutic Nucleic Acids composed of idoxuridine are degraded in the presence of fetal calf serum and uptaken by cells

Before the TNAs can mediate their proposed therapeutic effects, they need to be fragmented to free the biologically active monomeric nucleoside analogues (Figure 31, A). The release of the drug from the prodrug requires nucleases, which occur extra- and intracellularly. This process was examined by incubation of TNA-I_{14} in serum-free and serum-containing medium using HPLC quantification to track the intact TNA and the release of idoxuridine Alexander N. Zelikin, Aarhus University, Denmark). In serum-free medium TNA-I_{14} is not degraded for at least 24 h. This is in contrast to serum-containing medium, where TNA-I_{14} is degraded (Figure 31, B). Free idoxuridine was detected already after 2 h, while release efficiency further increased with longer reaction time (Figure 31, C). This leads to the assumption that TNAs are degraded into shorter oligomers by nucleases as well as phosphodiesterase in the FCS. Release of idoxuridine is most prominent between 2-6 h of incubation in serum-containing medium.

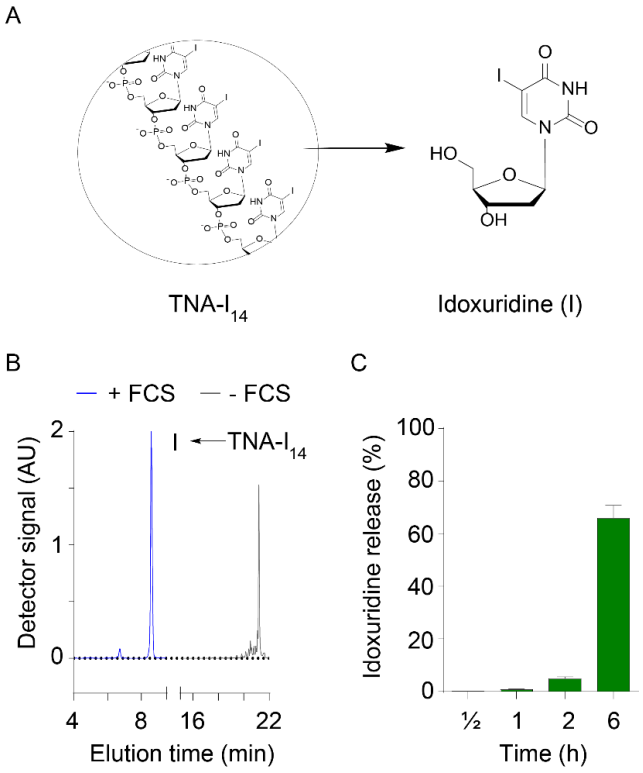

Figure 31: Degradation of TNA-I_{14} in cell culture medium with or without FCS over time.
A) Schematic illustration of the nuclease-mediated degradation of the TNA resulting in the release of idoxuridine. B) HPLC elution profiles for TNA-I_{14} after a 24 h incubation in cell culture medium with or without FCS. C) HPLC–based quantification of the idoxuridine release from TNA-I_{14} upon its incubation in serum-containing cell culture medium. Presented results are mean of three independent experiments ± ststandard deviation. Figure adapted from [186].

After confirming the degradation of TNAs, I was interested whether the TNAs are uptaken into the cells by using fluorescently labelled TNA-I_{14} and time-lapse confocal laser scanning microscopy. In human foreskin fibroblasts the uptake was observed after 20-30 min, for lung cancer cells A549 after 50-60 min, as shown in Figure 31 D. Since no difference between the uptake in presence or absence of serum was observed, an uptake independent from degradation of TNAs is suggested. Prior data on this subject showed that oligonucleotides of similar length exhibited efficient cell entry via endocytosis, possibly supported by specific membrane-bound proteins [120,212,405].

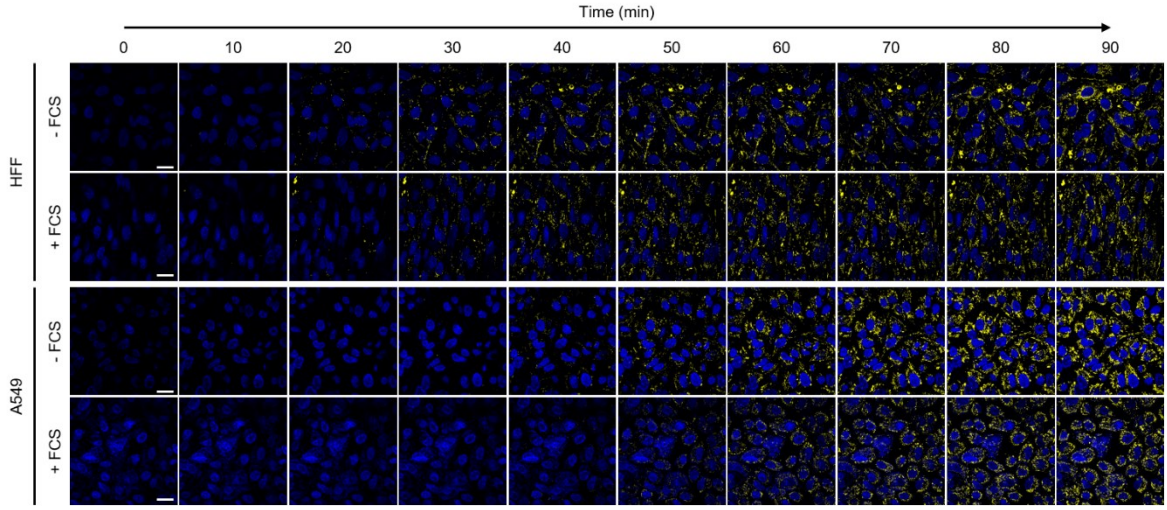

Figure 32: Time-lapse confocal laser scanning microscopy images illustrating cell entry of TNAs.
Cell entry of TNA-I_{14} in the presence of FCS (DMEM containing 10% FCS) or absence of FCS (X-VIVO™ medium) in human foreskin fibroblasts (HFF) and lung carcinoma cell line (A549) for 90 min. Picture of the same location was taken every 10 min. Nuclei were stained (blue) and TNA-I_{14}-Cy5 is false-colored in yellow. Scale bar is 20 µm (same for all panels). Figure adapted from [186].

3.5.3 Idoxuridine based Therapeutic Nucleic Acids are efficient inhibitors of HSV-1 and HSV-2

Next, the anti-HSV activity of the therapeutic nucleic acids (Table 5) was tested. For this, Vero E6 cells were incubated with the TNAs 2 h prior infection with HSV-2. I took advantage of the pronounced cytopathic effect induced by this virus and determined cell viability as an indirect measure of viral infectivity. Antivirals that inhibit HSV-2 infection would protect the cells from cytopathic effects, resulting in a metabolic activity that is not impaired. Furthermore, potential cytotoxic effects were determined by incubating the TNAs and controls with the cells in the same experimental set-up, but in the absence of virus (Figure 33).

All TNAs containing Idoxuridine showed significant inhibitory effects on HSV-2 infection without adversely affecting the cell viability (Figure 33 A, B and G). The control Idoxuridine also inhibited HSV-2 infection (Figure 33, I), but with lower efficiency compared to the TNAs. This is further highlighted in Table 6 showing the calculated IC_{50} values of all tested compounds. It is evident that the TNAs composed of idoxuridine are much more potent

compared to the monomeric drug itself. This effect has been described earlier for oligonucleotides. Specific receptor-mediated uptake lead to a greater pharmacological effect of the oligonucleotides than a non-specific uptake. This was even true for the detection of the same oligonucleotide amounts in the cells [166]. This is also in good agreement with the uptake studies Figure 32. TNAs composed of thymidine had no impact on the cell viability. Surprisingly, TNA-T_{14} also inhibited the HSV-2 infection, but only to a small extent (Figure 33, E and F). Unexpectedly, TNAs composed of Trifluridine (Figure 33, C, D and H) as well as the control Trifluridine (Figure 33, J) did not show antiviral activity. The curves for infection seem to increase, which was a results of the markedly decreased cell viability (Figure 33, C, D and J). Since HSV-2 is a cytopathic virus the metabolic activity of the cells was determined as read-out for the infection. Not infected cells that are affected by other toxic effects possibly will therefore be falsely determined as infected cells. Furthermore, besides the use as an anti-herpetic agent in earlier days Trifluridine is currently predominantly used for the treatment of metastatic colorectal cancer (Lonsurf®) [174]. $(T/F)_7$, the copolymer of alternating thymidine and trifluridine, had neither an impact on the infection nor on the cell viability, which might be due to the lower concentration of both individual parts.

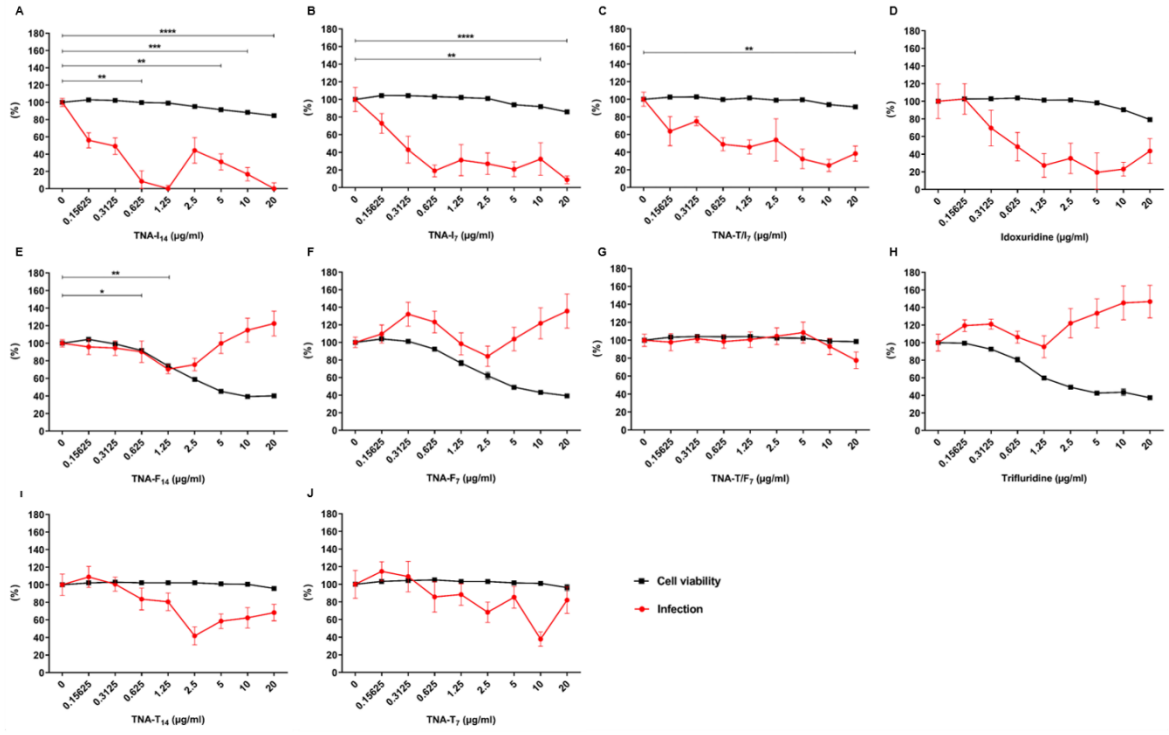

Figure 33: Dose response curves for TNAs in preventing HSV-2 infection of Vero E6 cells and corresponding cell viability data.
Cells were incubated with TNAs or controls, A) TNA-I_{14}, B) TNA-I_7, C) TNA-T/I_7, D) Idoxuridine, E) TNA-F_{14}, F) TNA-F_7, G) TNA-T/F_7, H) Trifluridine, I) TNA-T_{14} or J) TNA-T_7 for 2 h prior infection, and 72 h afterwards infection was determined by using CellTiter-Glo®(CTG). Percentage of infection was calculated by substracting sample values from uninfected controls and normalizing to the untreated controls (red lines). To determine the cell viability Vero E6 were incubated with TNAs for 74 h without infection. Data are normalized to the viability of cells in absence of the compounds (black lines). n = 3 in triplicates, ±SEM. *p< 0.1, **p< 0.01, ***p< 0.001 and ***p<0.0001 by one-way ANOVA with Bonferroni post-test. Figure adapted from [186].

Table 6: IC_{50} values of the tested therapeutic nucleic acids and controls on Vero E6 cells. Displayed are the IC_{50} values in µg/ml (calculated with GraphPad Prism), the molecular weight as well as the IC_{50} values in µM. n.a. = not available.

	T_{14}	F_{14}	$(T/F)_7$	I_{14}	$(T/I)_7$	I_7	Idoxuridine	Trifluridine
IC_{50} (mµ/ml)	11.62	n. a.	105.5	0.45	1.4	0.3	0.9	n. a.
MW (Da)	4486	5241	4863	6052	5269	3139	354	296
IC_{50} (µM)	2.59	n. a.	21.69	0.08	0.26	0.11	2.57	n. a.

A huge problem of antiviral drugs is the ability of viruses to develop resistances. Acyclovir is the antiviral drug for the treatment of HSV according to WHO [391], but also for this drug resistances can occur and mainly affect immunocompromised patients [15,206,265,292]. The resistance of the viruses to acyclovir results in 95% of cases from a frameshift mutation in the thymidine kinase [45,299]. The thymidine kinase is essential for the first phosphorylation step of the nucleoside analogue acyclovir, before cellular kinases take over to generate a triphosphate of the nucleoside analogue. This can than be incorporated into the viral DNA during replication process leading to a strand termination [15,99,340]. Idoxuridine is also dependent on the first phosphorylation step [67,363]. The TNAs are coupled via a phosphate at the site, where the phosphorylation by the thymidine kinase takes place [191]. Although the TNAs are degraded by a nuclease (Figure 30), it is unclear whether the resulting monomer is still phosphorylated or not due to lack of the HPLC and MALDI to detect the different monomers. Therefore, I was interested in the antiviral activity of the TNAs using clinical isolates of HSV-1 and HSV-2 with and without resistance to acyclovir.

Again, Vero E6 cells were incubated with the 4 different TNAs and Idoxuridine and Trifluridine as control for 2 h prior infection with the different clinical isolates of HSV-1 and HSV-2. The TNAs composed of Idoxuridine as well as Idoxuridine itself as control inhibit the non-resistant isolates of HSV-1and HSV-2. Unfortunately, this was not the case for the acyclovir resistant strains (Figure 34, A and B), since this indicates that the non-phosphorylated version is released after nuclease degradation. Compared to I_{14} (Figure 34, A) the trifluridine-based TNA F_{14} (Figure 34, E) has only a small influence on HSV infection. Trifluridine itself did not inhibit HSV infections (Figure 34, F). T_{14} (Figure 34, D) again exhibited, however still surprising, antiviral activity against the clinical isolates sensitive to acyclovir. This effect cannot be contributed to a polyanionic effect [361], which would rather impede the viral entry independent of an acyclovir resistance.

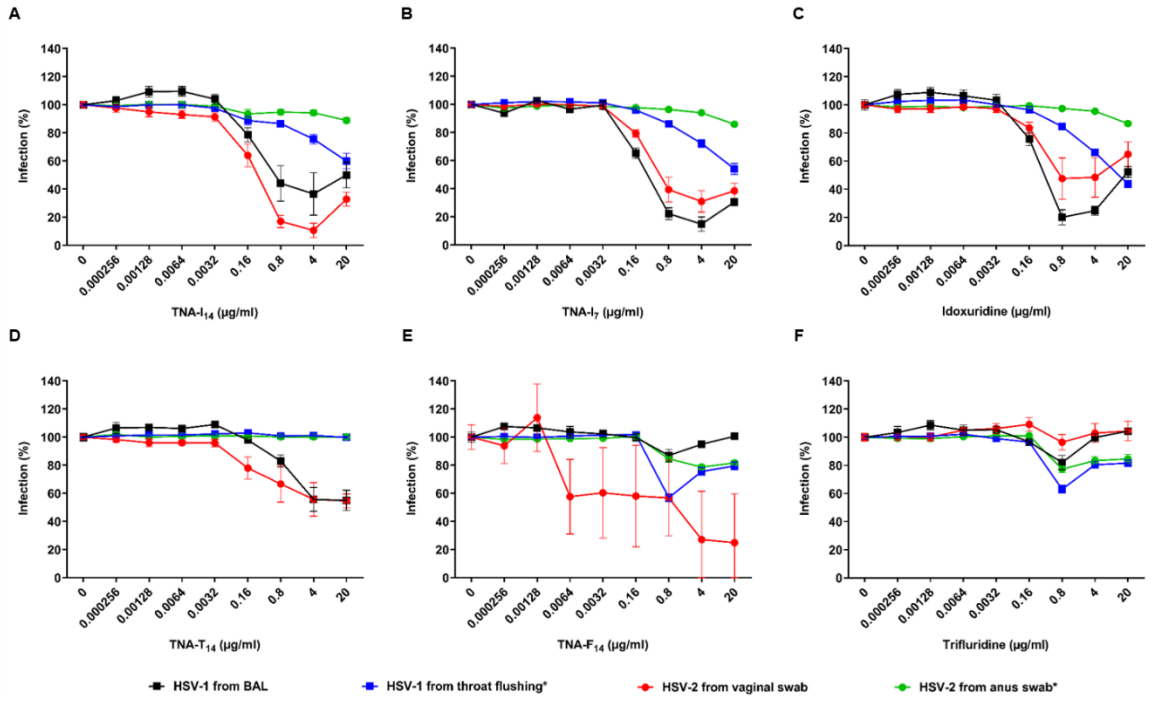

Figure 34: Dose response curves illustrating activity of TNA against HSV-1 and HSV-2 strains sensitive or resistant to acyclovir.
Vero E6 cells were incubated with TNAs or controls A) TNA-I_{14}, B) TNA-I_7, C) Idoxuridine, D) TNA-T_{14}, E) TNA-F_{14} or F) Trifluridine for 2 h prior infection. Infection was performed with HSV-1 isolates from broncho-alveolar lavage (BAL) and from throat flushing as well as with HSV-2 isolates from vaginal swab and anus swab. Isolates indicated with * are acyclovir resistent. 72 h later infection rates were determined by using the MTT cell viability assay. Percentage of infection was calculated by substracting sample values from uninfected controls and normalizing to the untreated controls. n = 3 in triplicates, ±SEM. Figure adapted from [186].

Besides measuring the antiviral activity based on a cell viability assay the experiment was analyzed microscopically (Figure 35 and Figure 36). Herpes simplex viruses are cytopathic viruses leading to morphological changes of the cell expressed as rounded cells or in later stage to multinuclear cells (syncytia) [105,204,383]. The rounding and detachment of cells in untreated controls is obvious since the cells appear as darker spots comparing to uninfected cells were the cells are still confluent (Figure 35 and Figure 36). Idoxuridine-based TNAs reduce viral infectivity and rescue the cells from infection-induced cytopathic effects (confluency of cells is comparable to non-infected cells) by non-acyclovir resistant strains (Figure 35, A and Figure 36, A). Additionally, this effect was seen partially for TNA-F_{14} and TNA-T_{14}. In case of the acyclovir-resistant drugs, no rescue from cytotoxic effects caused by the viruses was achieved (Figure 35, B and Figure 36, B). This is in line with the results shown in Figure 34. Conclusively, this means that only the de-phosphorylated version of the drug was present. In consequence this form was not phosphorylated by the viral thymidine kinase, which was deficient in the tested acyclovir-resistant strains.

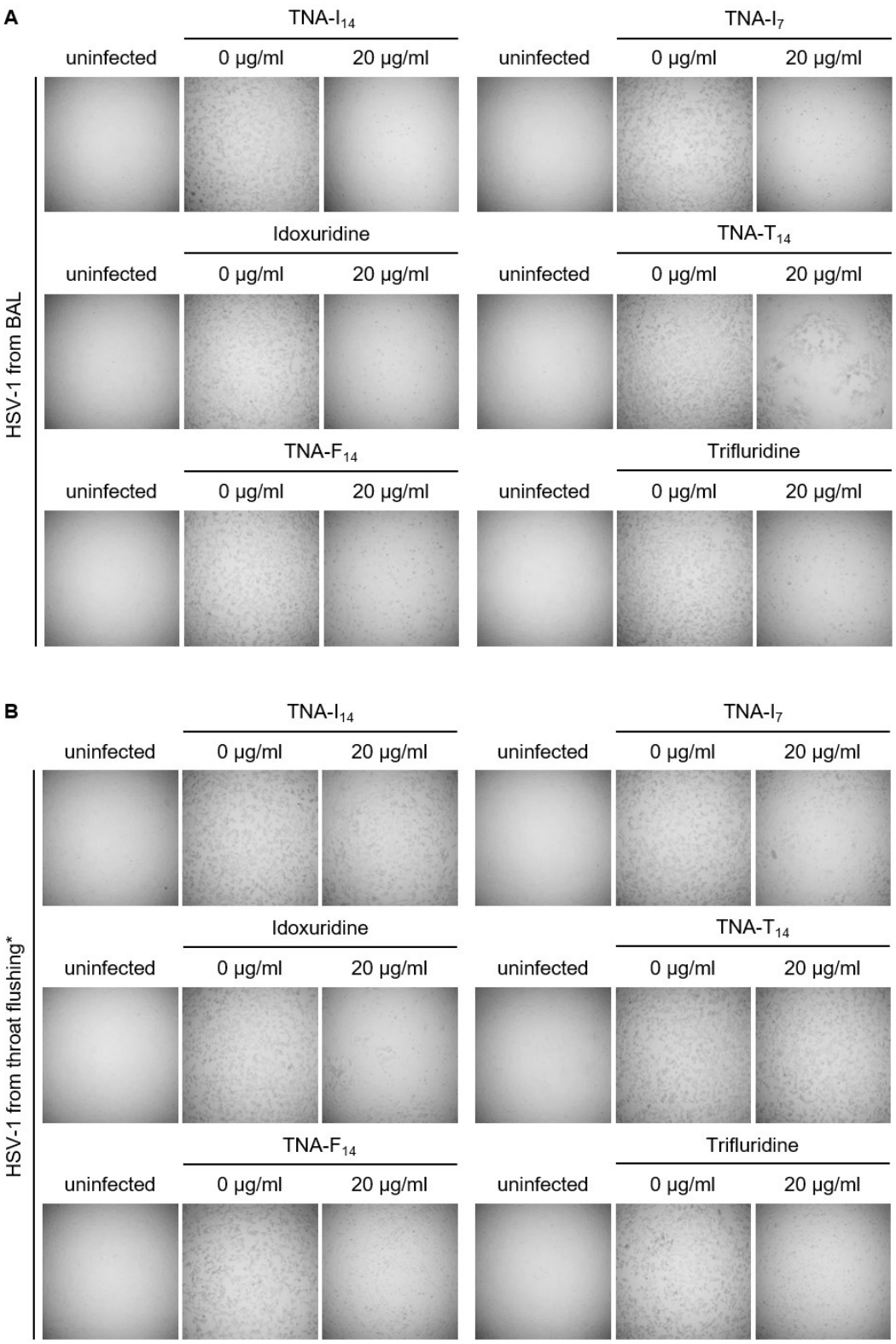

Figure 35: Bright field microscopy images illustrating antiviral effect of TNAs upon infection of Vero E 6 cells with HSV-1 isolates.

Vero E6 cells were treated with different TNAs and controls (Idoxuridine and Trifluridine) 2 h prior infection with either A) HSV-1 from broncho-alveolar lavage (BAL) or B) HSV-1 from throat flushing. * indicates acyclovir resistance. Shown are uninfected cells and cells exposed to 20 µg/ml of respective compound in presennce of the indicated virus. Bright field images were taken with a Leica MC120 HD color camera connected to a microscope with a 10x objective. Figure adapted from [186].

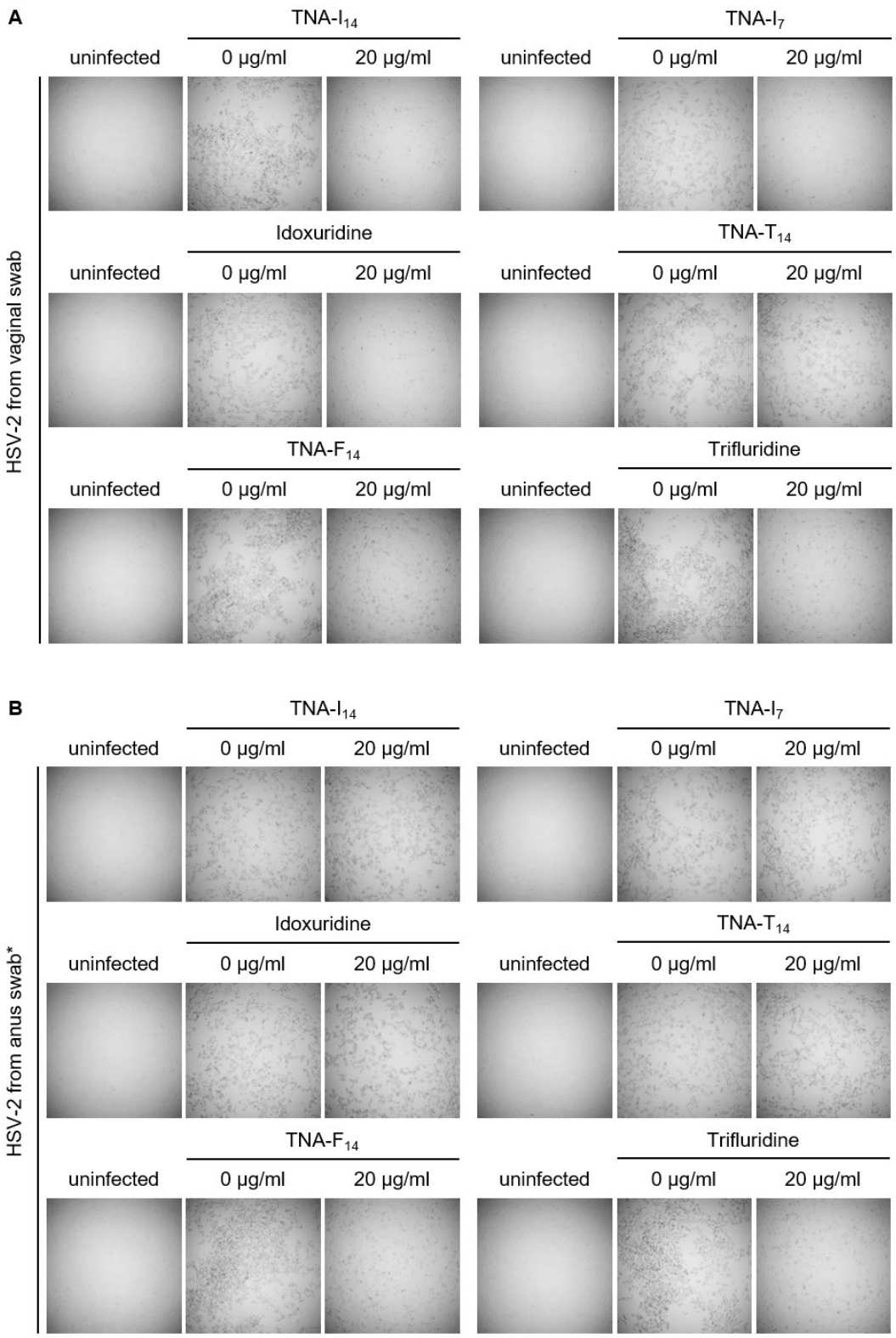

Figure 36: Bright field microscopy images illustrating antiviral effect of TNAs upon infection of Vero E 6 cells with HSV-2 isolates.

Vero E6 cells were treated with different TNAs and controls (Idoxuridine and Trifluridine) 2 h prior infection with either A) HSV-2 from vaginal swab or B) HSV-2 from anus swab. * indicates acyclovir resistance. Shown are uninfected cells and cells exposed to 20 µg/ml of respective compound in presennce of the indicated virus. Bright field images were taken with a Leica MC120 HD color camera connected to a with a 10x objective. Figure adapted from [186].

To further exclude that the dephosphorylation of the TNA is a result of the presence of FCS, the effect of the TNAs on clinical isolates of HSV-2 were further investigated in chemical defined medium without FCS. Nearly identical inhibition profiles in the presence (Figure 34) or absence of FCS (Figure 37) was achieved. Again, only the non-acyclovir resistant strains was inhibited by TNA-I_{14}, TNA-I_7 and Idoxuridine (Figure 37, A-C). The effect of TNA-F_{14} and Trifluridine (Figure 37, E and F) was a result of the previously determined cytotoxic effect of the drug itself (Figure 33). TNA-T_{14} did not had any effect on the viral infectivity (Figure 37, D). The results reveal that intracellular degradation of TNAs lead to de-phosphorylated versions of the monomer, meaning the parent drugs Idoxuridine or Trifluridine.

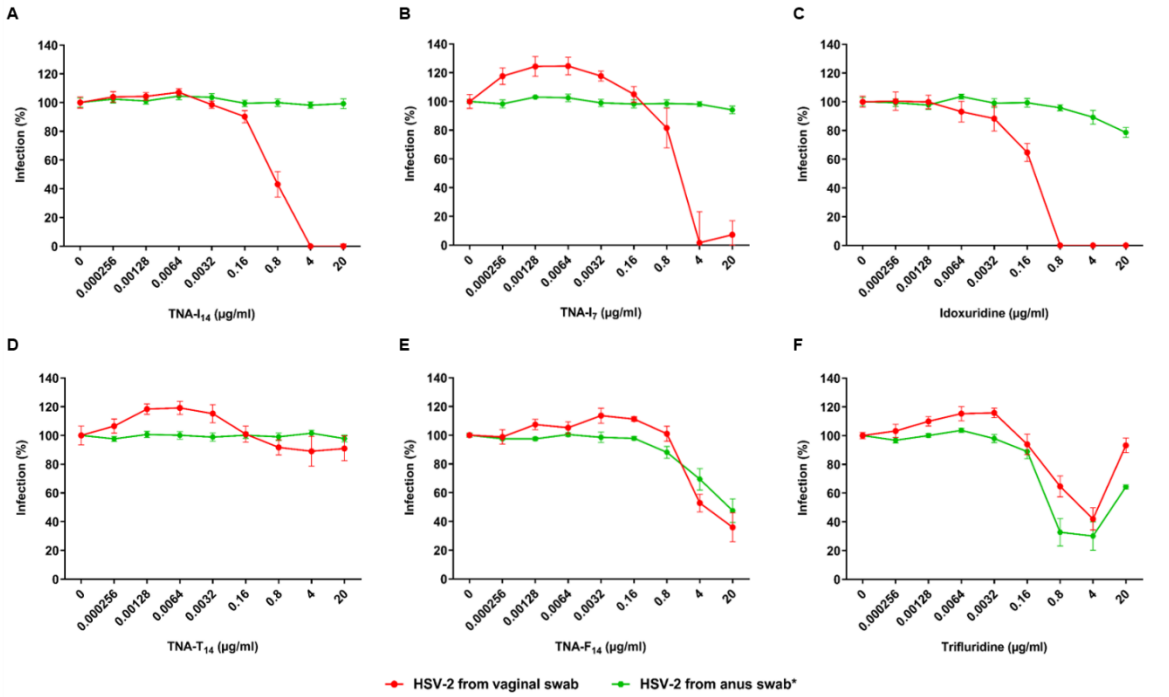

Figure 37: Dose response curves illustrating activity of TNAs against acyclovir sensitive or resistant clinical isolates of HSV-2.
Vero E6 cells were cultured in normal medium for one day. Before incubation of TNAs with Vero E6 cells the medium was discarded, cells were washed and then exposed to chemically defined medium without FCS. TNAs or controls or controls A) TNA-I_{14}, B) TNA-I_7, C) Idoxuridine, D) TNA-T_{14}, E) TNA-F_{14} or F) Trifluridine were incubated with the cells 2 h prior infection with HSV-2 from vaginal swab or anus swab. * indicates acyclovir-resistant iisolate. 72 h later infection rates were determined by using the MTT cell viability assay. Percentage of infection was calculated by substracting sample values from uninfected controls and normalizing to the untreated controls. n=2 in triplicates, ± SD. Figure adapted from [186].

In most of the assays monkey-derived Vero E6 were used. Therefore, I analyzed the effect of the TNAs in human cell lines infected with non-acyclovir resistant clinical isolates of HSV-1 (from BAL) and HSV-2 (from vaginal swab). Confocal laser scanning microscopy was used to visualize the cellular uptake and subsequent antiviral effect of the TNA-I_{14} and -F_{14} labelled with a Cy5-fluorophore. As observed earlier, F_{14} was slightly toxic to the cells and furthermore not able to prevent infection-induced changes in the morphology of A549 or HFF cell (Figure 38). In contrast, I_{14} rescued the cells from cytopathic effects without triggering any cytotoxic effects. Furthermore, this analysis confirmed the previously observed cellular uptake of TNAs (Figure 32).

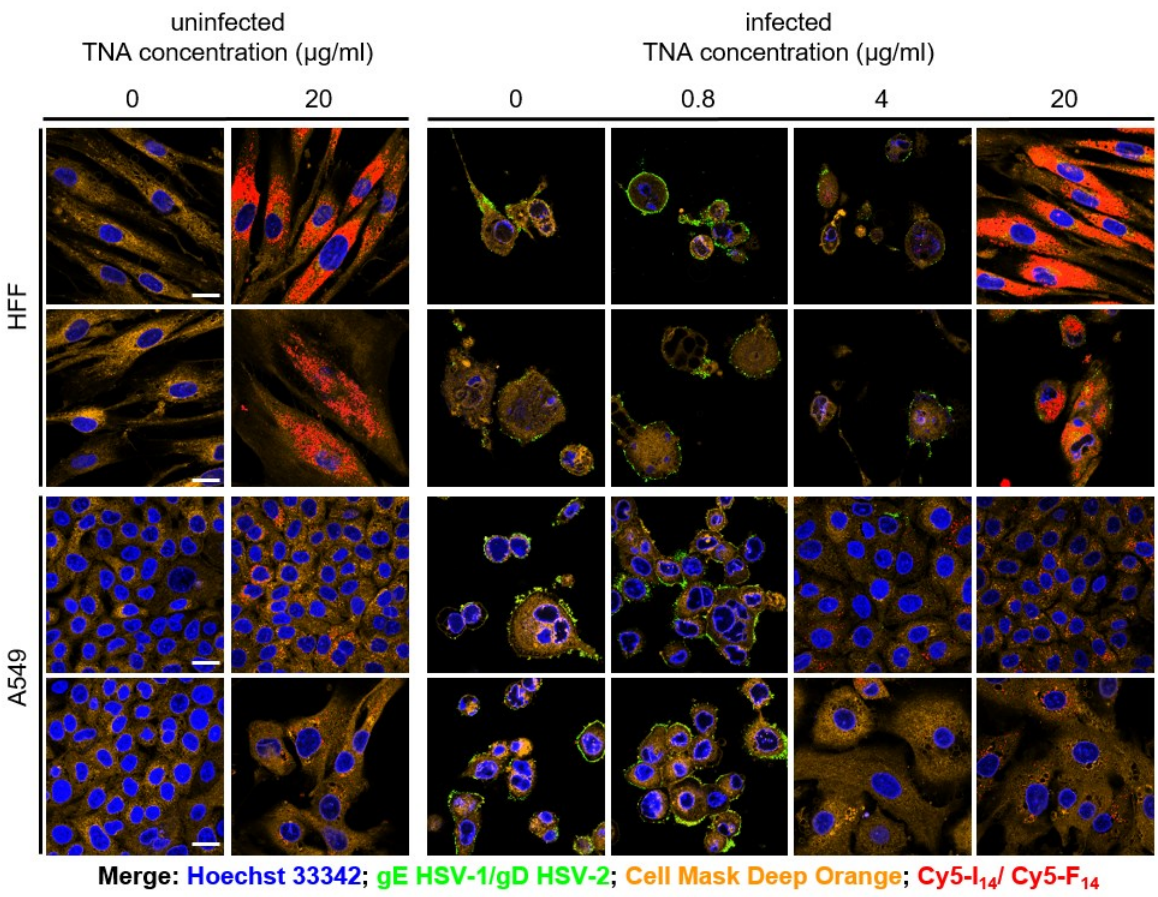

Figure 38: Antiviral effect of TNAs in A549 cells infected with HSV-1 and HFF cells infected with HSV-2.
HFF cells and A549 cells were incubated with Cy5-I_{14} and Cy5-F_{14} 2 h prior infection with HSV-1 from bronchoalveolar lavage (A549 cells) or HSV-2 from vaginal swab (HFF). Cells were fixed and stained for nuclei (blue), HSV glycoproteins (green) and plasma membranes (orange). The nucleic acids are coupled to the fluorophore Cy5 (red). Scale bar is 20 µm (same for all panels). Figure adapted from [186].

3.6 Therapeutic Nucleic Acids of nucleoside analogues as new treatment opportunity - Discussion

The first antiviral drug approved was Idoxuridine. It is a nucleoside analogue of thymidine and incorporated into the DNA during viral replication, which leads to a strand termination. This drug was used to treat HSV infections, but due to its unspecificity it lead to severe adverse effects and was only used in the treatment of keratoconjunctivitis [381]. Furthermore, this drug suffered from a low bioavailability as many other nucleoside analogues used for the treatment of HSV infections [362]. To improve the treatment of HSV infections macromolecular prodrugs are useful. The properties of nucleic acids such as high stability, defined sequence and controlled length is advantageous in the design of macromolecular prodrugs (MPs). Many other techniques suffer from their less controllable reaction, leading to batch-to-batch variations [236,424], which can have an influence on the safety and efficiency of a pharmaceutical product [94]. Therefore, synthesis of nucleic acids from nucleoside analogues such as Idoxuridine where performed, which resulted in highly defined molecular product composition with a precise number of nucleobases [41]. This is a highly desired outcome and crucial for potential future regulatory drug approval [353]. Furthermore, it was shown that the release of the monomeric drug components is a natural mechanism accomplished by nucleases. This mechanism is supposed to be rather intracellularly than extracellularly since the uptake of TNAs was much faster compared to the scission of the TNAs. The efficient and rapid uptake of the TNAs by the cells is in good agreement with previous published data showing an efficient cell entry of oligonucleotides of similar length via endocytosis, possibly due to specific membrane-bound proteins [120,212,405]. The uptake of the TNAs and the intracellular processing is shown in Figure 39.

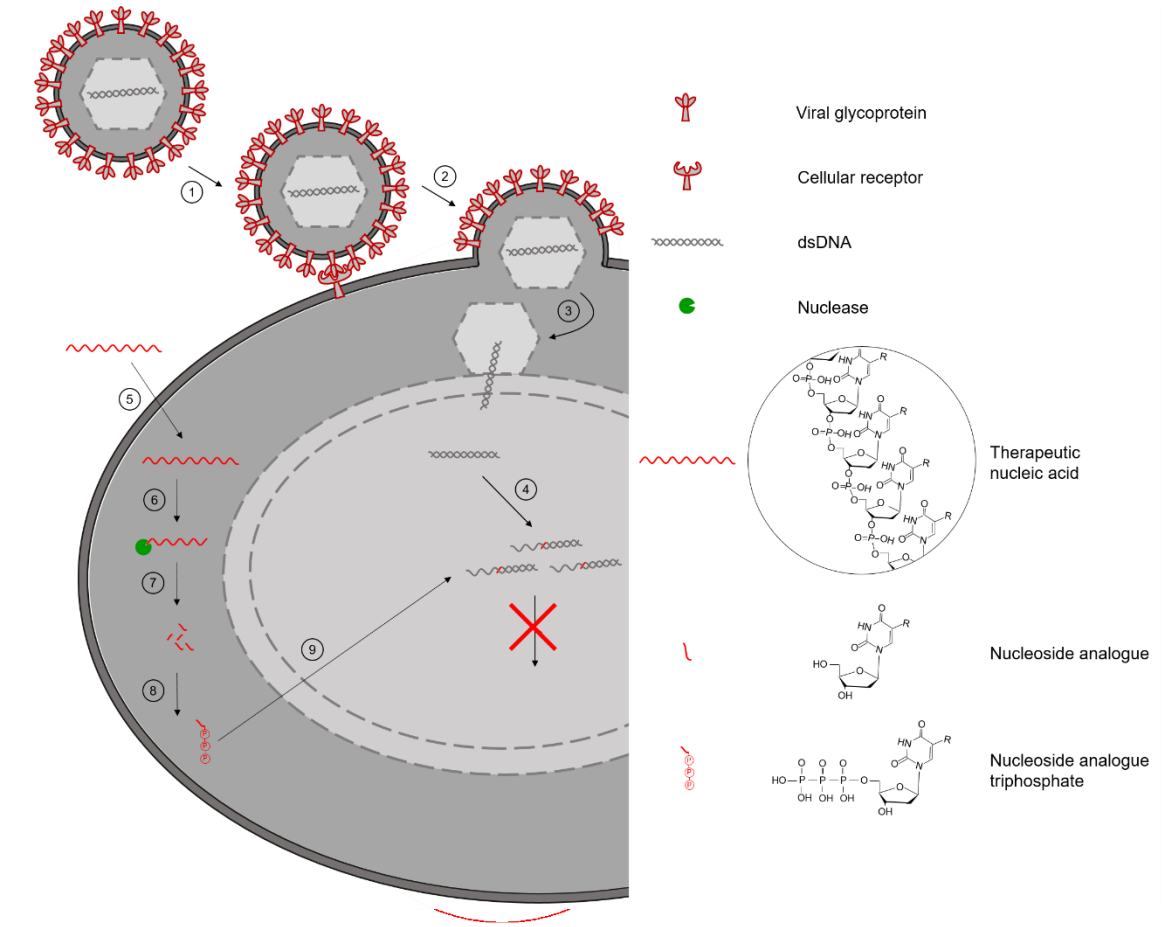

Figure 39: Schematic illustration of the mode of action of therapeutic nucleic acids.
1) HSV attaches to the cell surface via interaction of viral glycoportein with cellular receptors. 2) Viral envelope fuses with the plasma membrane. The capsid is released into the cytoplasm and capsid is transported to the nuclear pore. 4) Viral DNA is released into the nucleus and replication of the DNA starts. 5) Therapeutic nucleic acids are uptaken by the cell. 6) Nucleases degrade the therapeutic nucleic acids 7) into their respective monomers. 8) Three phoyphorylation steps lead to the activation of the monomers. 9) Triphosphorylated monomers are transported into the nucleus and incorporated into the replicating viral DNA. This leads to the strand termination and the inhibition of HSV.

TNAs composed of trifluridine indeed showed antiviral activity, but this was accompanied with anti-proliferative activity. Since trifluridine is not only used as an anti-herpetic agent, but also for the treatment of metastatic colorectal cancer [174] this observation was not surprising. This possibly will be used as another approach, where TNAs of Trifluridine are used to treat colorectal cancer more efficiently by providing a higher payload of the drug.

Thymidine-derived TNAs also showed an impact on HSV infections. The antiviral effect of the co-polymers with trifluridine or idoxuridine arose mostly from trifluridine and idoxuridine. T_{14} itself also showed anti-herpetic effects, but only to a minor extent, without impairing the cell viability. Since all TNAs only had an impact on acyclovir-sensitive strains, the effect of T_{14} cannot be explained by its polyanionic nature [361]. This would be a sequence-independent mechanism and all viruses should have been inhibited.

TNAs composed of idoxuridine showed to be potent and efficacious antiviral agents. TNA of the 14-mer and 7-mer showed a 35-fold superior effect compared to the parent drug idoxuridine. The superior effect of I_{14} and I_7 points again to the uptake and intracellular scission, otherwise only matched potency of extracellular degraded TNAs compared to the

parent drug would be assumed. Furthermore, the uptake mechanism may also contribute to the increased potency of Idoxuridine-based TNAs, as described earlier for oligonucleotides [166].

Unfortunately, all tested TNAs were not able to inhibit clinical isolates of acyclovir-resistant HSV strains. The degradation of TNAs releases de-phosphorylated versions of the nucleoside analogue. However, mono-phosphorylated versions would be required to overcome the lack of activity of the viral thymidine kinase due to a mutation [45,299]. One approach therefore would be to create TNAs that release mono- or even di- or trisphorylated versions of a nucleoside analogue that has high affinity to be incorporated into viral DNA.

All in all, the TNAs are macromolecular prodrugs that facilitate a significant amount of deliverable payload [134]. They are composed of nucleobases which was also used in a combinatory therapy as shown by the possibility to synthesize co-polymers. Furthermore, they can be used for long-acting repertoire of drugs since materials and methods to deliver nucleic acids already exist and therefore can be easily adapted [41].

4 Summary

Until now, there are many antiviral drugs on the market but only selected viruses are targeted. In many cases, drugs are often invented in the aftermath of an outbreak and only the symptoms are treated. To prevent epidemic outbreaks of viruses broad-spectrum antiviral agents are highly appealing. Macromolecular prodrugs consisting of polymeric anions were generated and studied to address these unmet medical needs. Poly(vinylbenzoic acid) (PVBzA), was the only compound that inhibited all tested enveloped viruses. PVBzA is a compound with an anionic as well as hydrophobic character. It was shown, that both characteristics are necessary to inhibit viruses. The proposed mode of action is the interaction with viral glycoprotein and an increased electrostatic repulsion and therefore inhibiting the viral attachment. This can be an attempt for the use as microbicide in antiviral gels, sprays, or coatings for diverse biomedical and biotechnological applications.

Beyond a direct antiviral activity, macromolecular complexes are promising tools to optimize the delivery and to improve the pharmacokinetics of already existing drugs. Therefore, macromolecular prodrugs of acyclovir were developed. Acyclovir was conjugated via a self-immolative linker to a polymer to achieve high payload. The polymers linked to a fatty acid bind albumin and thereby increase the half-life of acyclovir, which is of special interest for injections. Those macromolecular prodrugs showed *in vitro* as well as *in vivo* efficacy by protecting mice from Herpes simplex virus type 2 (HSV-2) infections after subcutaneous administration prior to vaginal infection. Formulations, such as 1,2-distearoyl-sn-glycero-3-phosphoethanolamine poly(N-(2-hydroxypropyl)methacrylamide) acyclovir (DSPE-PHPMA-ACV) can non-covalently bind to albumin and can reduce the administration by prolonging the duration of the drugs.

Another antiviral treatment approach were macromolecular prodrugs based on natural nucleic acid scaffolds. Nucleoside analogues, specifically idoxuridine and trifluridine were used to synthesize molecularly defined therapeutic nucleic acids (TNA). TNAs were efficiently taken up by cells, followed by nuclease-mediated degradation which releases the active monomers. Especially idoxuridine-containing TNAs showed antiviral activity against Herpes simplex virus type 1 (HSV-1) and HSV-2, with a superior potency compared to the parent drug. Therefore, TNAs appear to be of high interest due to their natural degradation, the controllable composition, which can also be used in combinatorial therapies and their superior potency.

Altogether, macromolecular prodrugs might be tested as novel concepts to treat HSV-2 and other enveloped viruses and therefore represent interesting tools for antiviral therapies regarding their high payload, their modifiability e.g. for increasing the body half-life and their possibility to be used as broad-spectrum microbicides.

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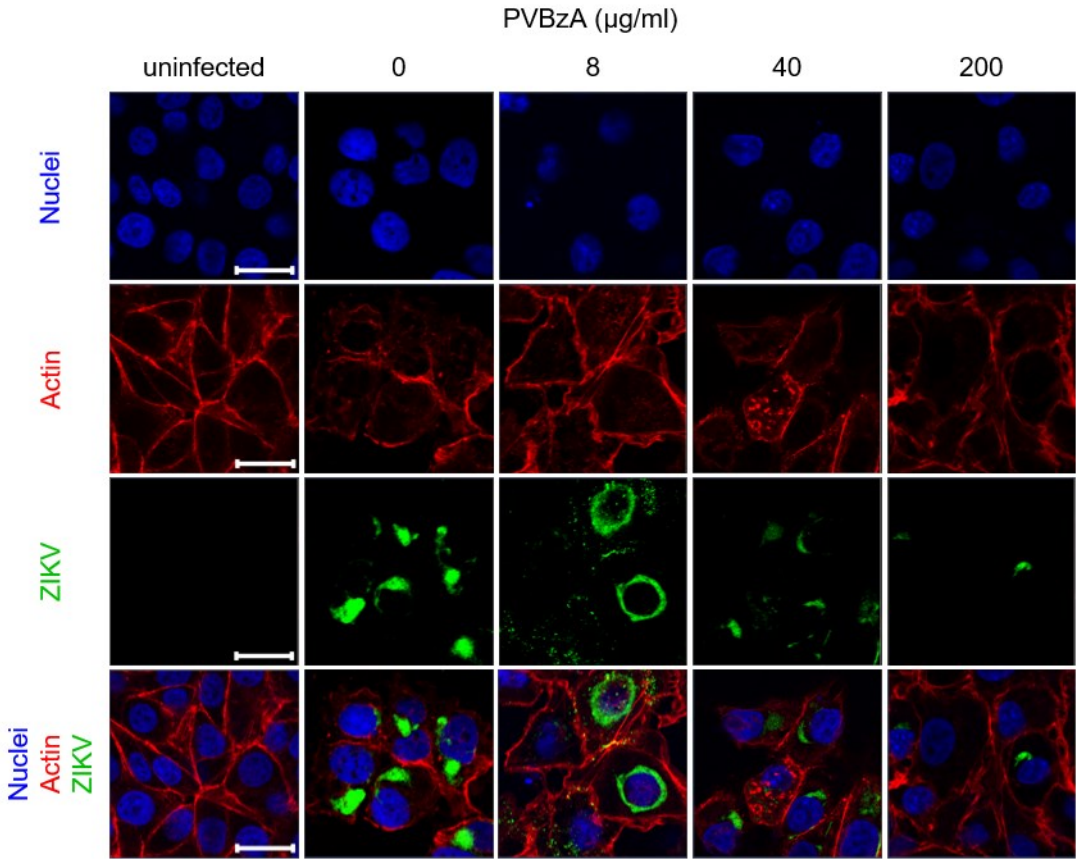
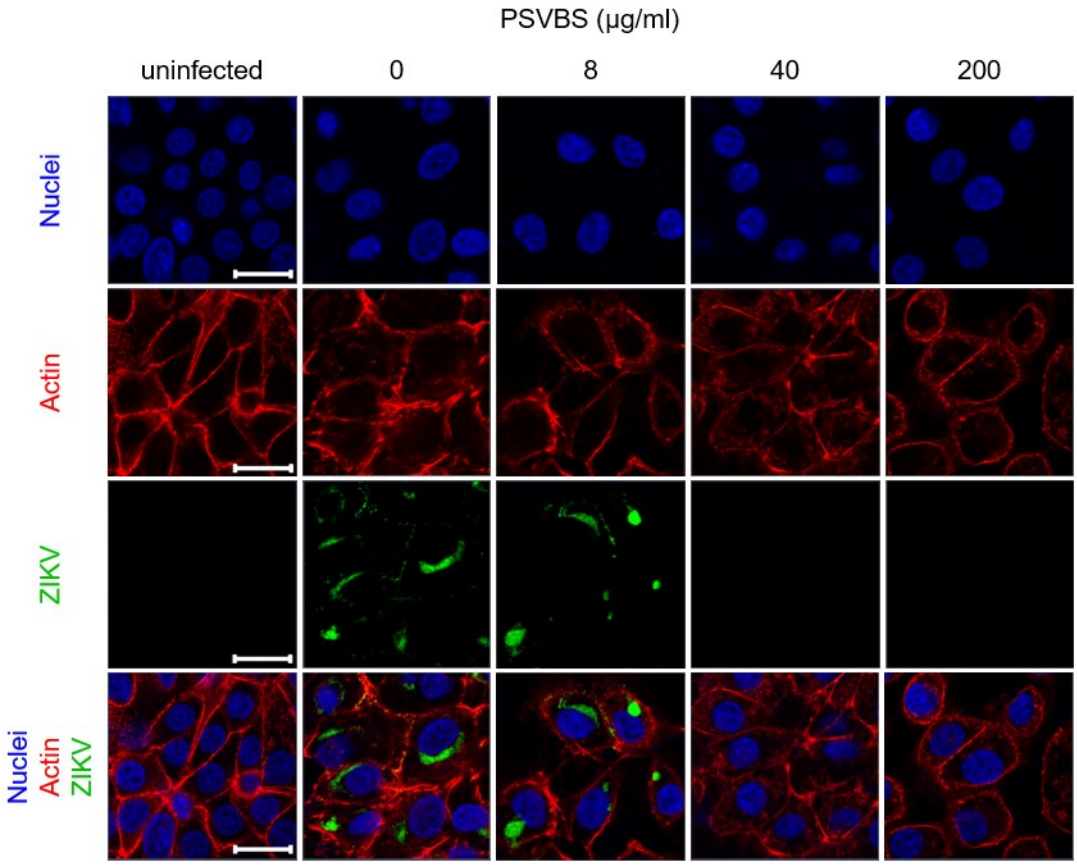
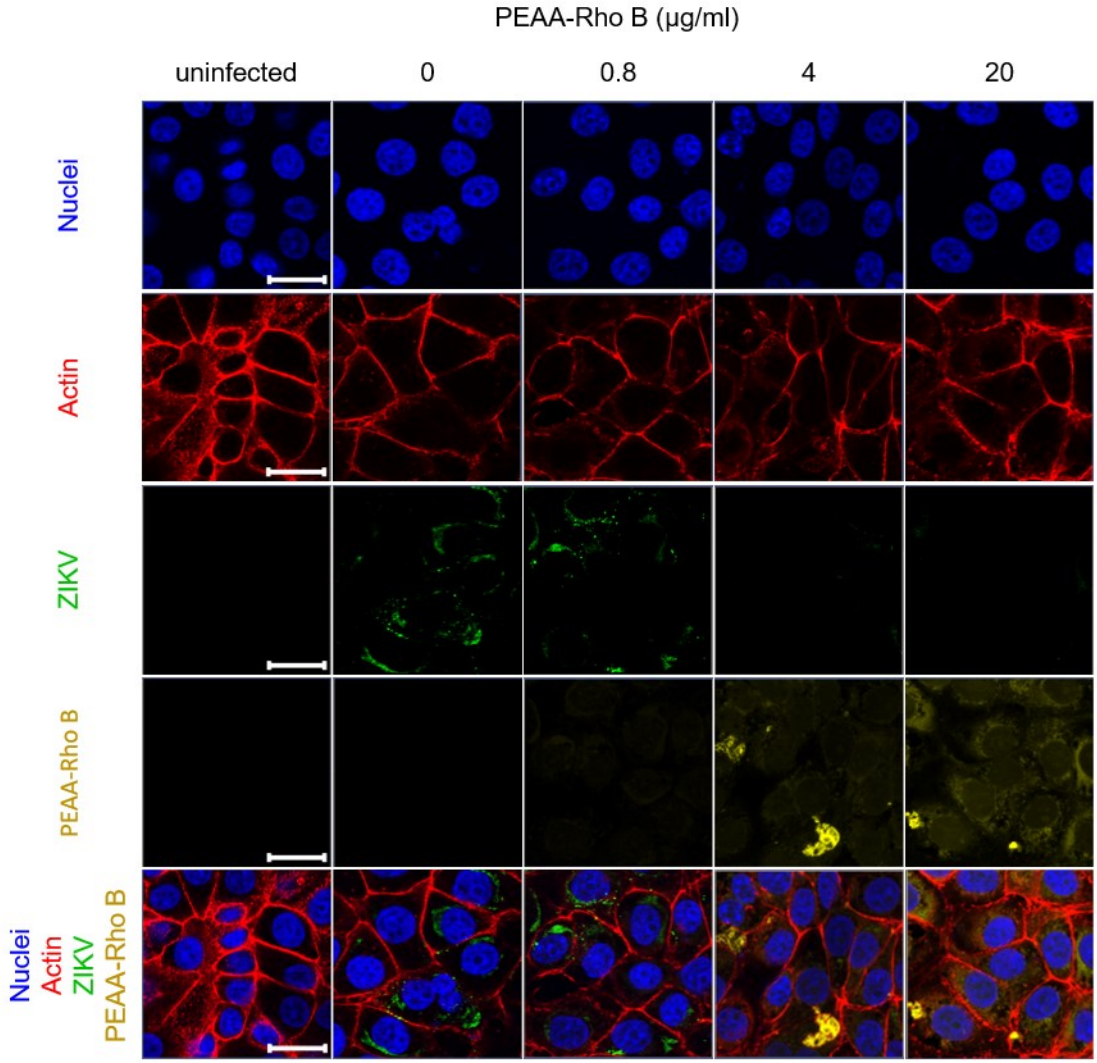
6 Appendix



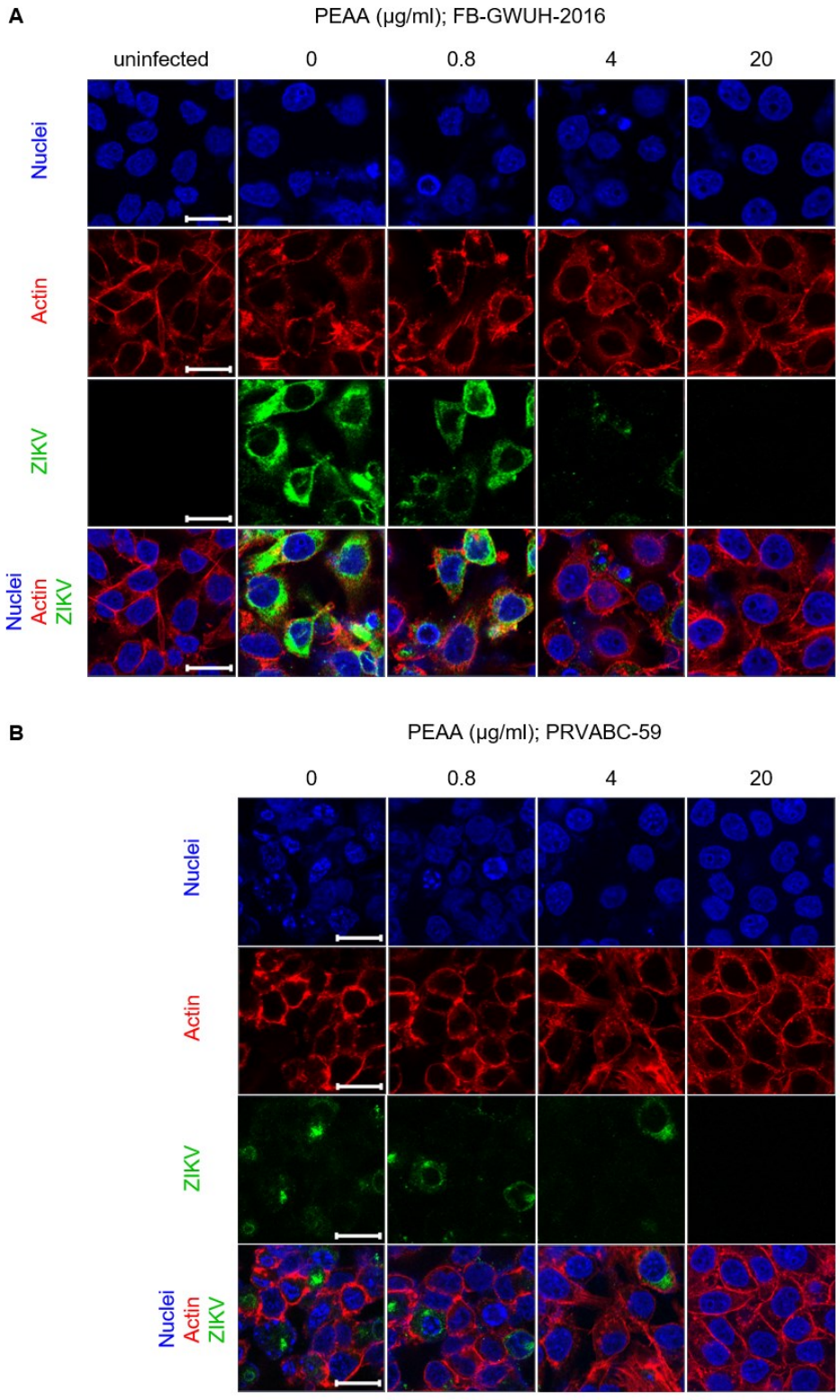
Supplementary figure 1: PVBzA inhibts ZIKV MR766 infection in Vero E6 cells under cell treatment conditions.
Vero E6 clles were incubated with PVBzA 90 min prior infection with ZIKV MR766. Three days later cells were fixed and stained for protein E (green), nuclei (blue) and actin (red). Shown are exemplary confocal images. Scale bars correspond to 20 µm. Figure adapted from [300], Copyright Wiley-VCH Verlag GmbH & Co. KgaA. Reproduced with permission.



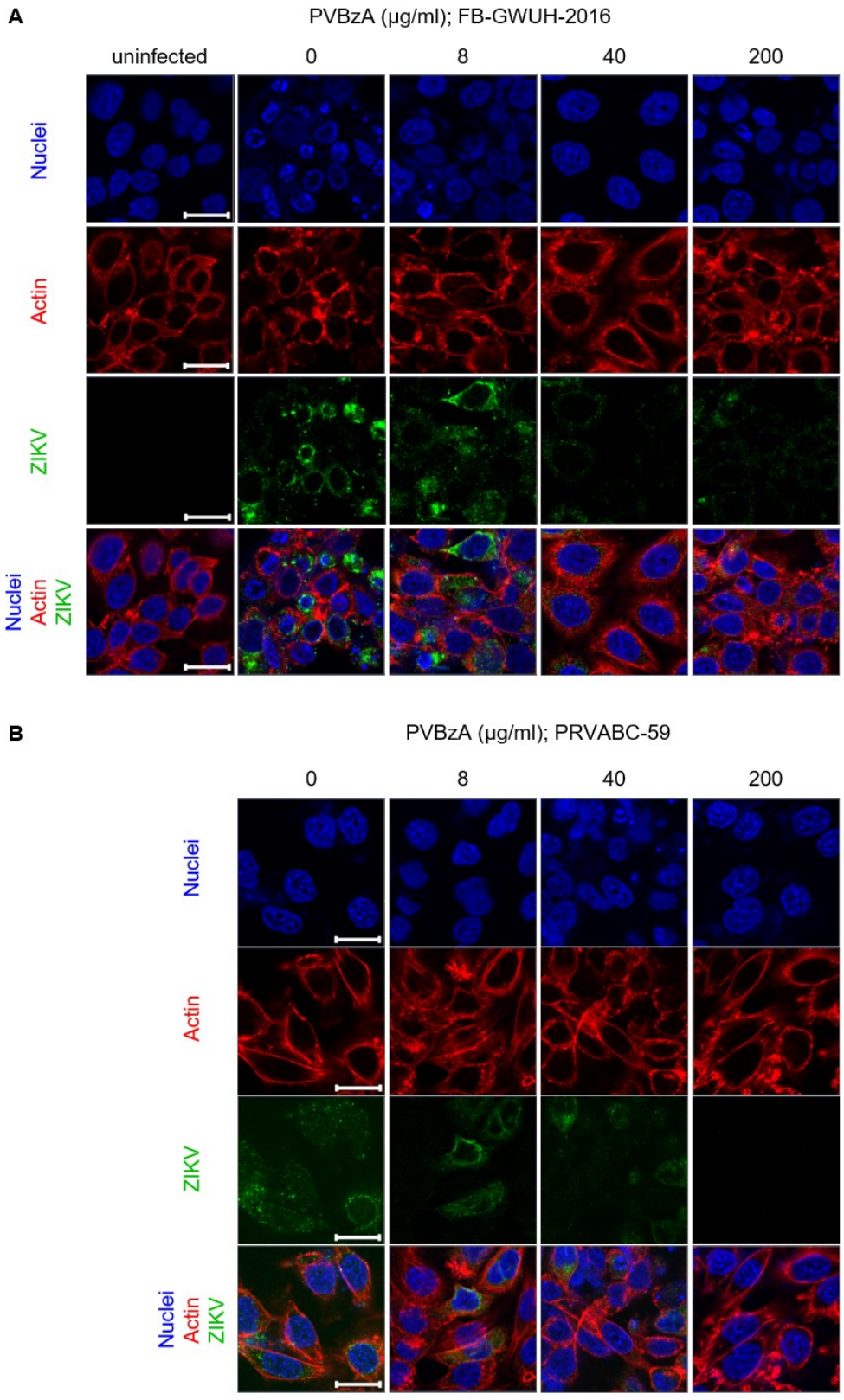
Supplementary figure 2: PSVBS inhibts ZIKV MR766 infection in Vero E6 cells under cell treatment conditions.
Vero E6 clles were incubated with PSVBS 90 min prior infection with ZIKV MR766. Three days later cells were fixed and stained for protein E (green), nuclei (blue) and actin (red). Shown are exemplary confocal images. Scale bars correspond to 20 µm. Figure adapted from [300], Copyright Wiley-VCH Verlag GmbH & Co. KgaA. Reproduced with permission.



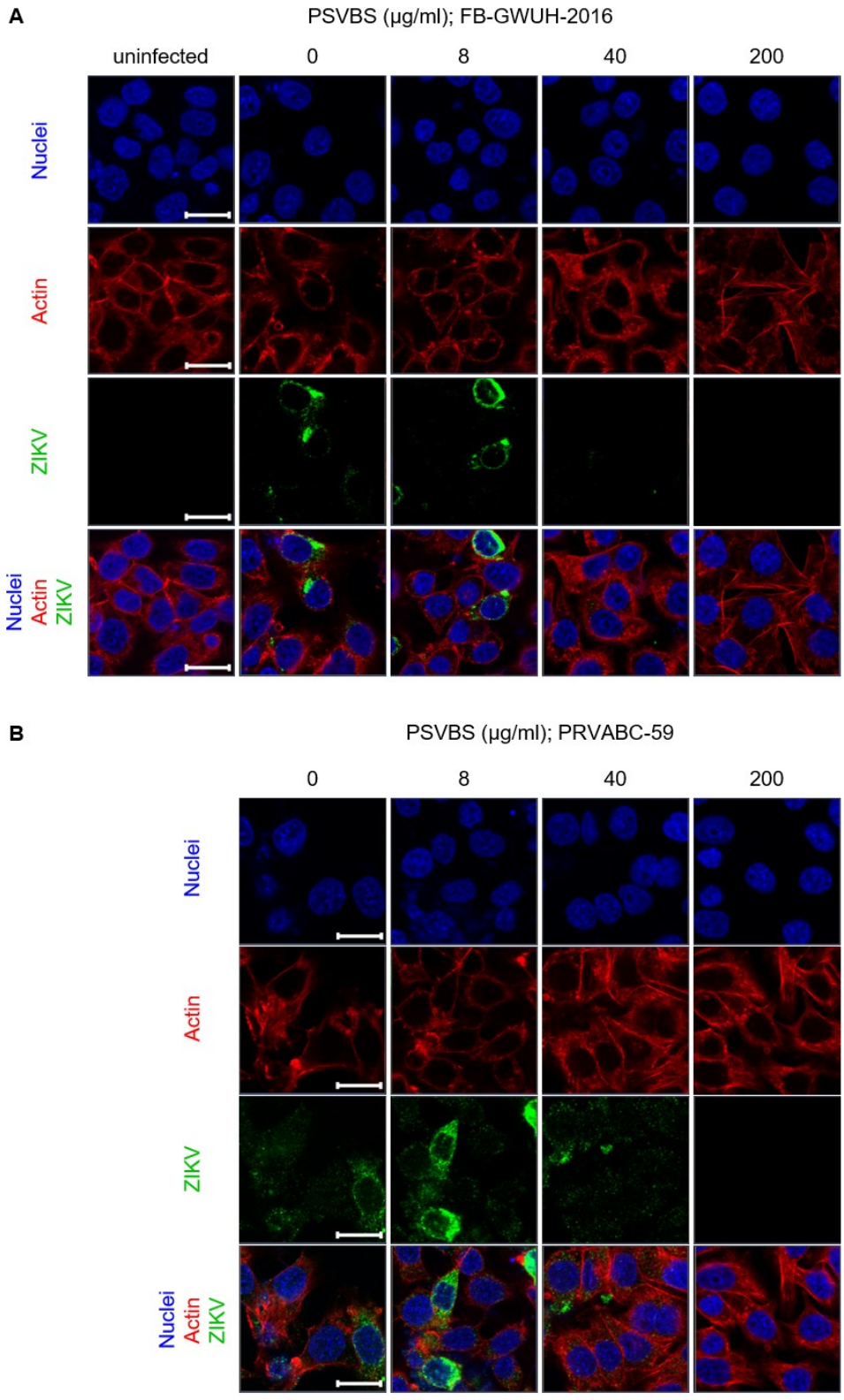
Supplementary figure 3: PEAA-Rho B inhibits ZIKV MR766 under virion treatment conditions.
MR766 was inoculated with PEAA-Rho B for 15 min prior infection of Vero E6 ells. Three days later cells were fixed and stained for protein E (green), nuclei (blue) and actin (red). Shown are exemplary confocal images. Scale bars correspond to 20 µm (same for all panels). Figure adapted from [300], Copyright Wiley-VCH Verlag GmbH & Co. KgaA. Reproduced with permission.



Supplementary figure 4: PEAA inhibits clinically relevant ZIKV isolates in HeLa cells under virion treatment conditions.
A) FB-GWUH-2016 or B) PRVABC-59 was inoculated with PEAA for 15 min prior infection of HeLa cells. Three days later cells were fixed and stained for protein E (green), nuclei (blue) and actin (red). Shown are exemplary confocal images. Scale bars correspond to 20 µm (same for all panels). Figure adapted from [300], Copyright Wiley-VCH Verlag GmbH & Co. KgaA. Reproduced with permission.



Supplementary figure 5: PVBzA inhibits clinically relevant ZIKV isolates in HeLa cells under cell treatment conditions.
HeLa cells were incubated with PVBzA for 90 min prior infection with with A) FB-GWUH-2016 or B) PRVABC-59 for 15 min prior infection of HeLa cells. Three days later cells were fixed and stained for protein E (green), nuclei (blue) and actin (red). Shown are exemplary confocal images. Scale bars correspond to 20 µm (same for all panels). Figure adapted from [300], Copyright Wiley-VCH Verlag GmbH & Co. KgaA. Reproduced with permission.



Supplementary figure 6: PSVBS inhibits clinically relevant ZIKV isolates in HeLa cells under cell treatment conditions.
HeLa cells were incubated with PSVBS for 90 min prior infection with with A) FB-GWUH-2016 or B) PRVABC-59 for 15 min prior infection of HeLa cells Three days later cells were fixed and stained for protein E (green), nuclei (blue) and actin (red). Shown are exemplary confocal images. Scale bars correspond to 20 µm (same for all panels). Figure adapted from [300], Copyright Wiley-VCH Verlag GmbH & Co. KgaA. Reproduced with permission.

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PUBLICATIONS

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