Macromolecular Antiviral Agents against Zika, Ebola, SARS, and Other Pathogenic Viruses

Franziska Schandock, Camilla Frich Riber, Annika Röcker, Janis A. Müller, Mirja Harms, Paulina Gajda, Kaja Zuwala, Anna H. F. Andersen, Kaja Borup Løvschall, Martin Tolstrup, Florian Kreppel, Jan Münch, and Alexander N. Zelikin**

Viral pathogens continue to constitute a heavy burden on healthcare and socioeconomic systems. Efforts to create antiviral drugs repeatedly lag behind the advent of pathogens and growing understanding is that broad-spectrum antiviral agents will make strongest impact in future antiviral efforts. This work performs selection of synthetic polymers as novel broadly active agents and demonstrates activity of these polymers against Zika, Ebola, Lassa, Lyssa, Rabies, Marburg, Ebola, influenza, herpes simplex, and human immunodeficiency viruses. Results presented herein offer structure–activity relationships for these pathogens in terms of their susceptibility to inhibition by polymers, and for polymers in terms of their anionic charge and hydrophobicity that make up broad-spectrum antiviral agents. The identified leads cannot be predicted based on prior data on polymer-based antivirals and represent promising candidates for further development as preventive microbicides.

1. Introduction

Zika virus (ZIKV) represents the latest in the series of viral pathogens that recently (re-)emerged to become a global healthcare problem. Indeed, spanning the entire history of humankind and to current days, viral pathogens constitute an enormous socioeconomic burden. Some viruses have high associated mortality rates, as is the case for Ebola virus, middle east respiratory syndrome (MERS), or severe acute respiratory syndrome (SARS) coronavirus.[1] Other viruses are not life-threatening but represent a major cause for morbidity. For these pathogens, greatest burden is that of containment and limiting the spread of the virus. Successes of medicinal chemistry in the design of antiviral agents are becoming more and more sound. Currently, 90 antiviral drugs categorized into 13 functional groups have been approved for the treatment of human immunodeficiency virus type 1 (HIV-1), herpes simplex viruses (HSV) types 1 and 2, influenza, varicella zoster, hepatitis B and C, papilloma, and respiratory syncytial viruses.[2–4] However, against the majority of viruses no treatment option exists. In addition, the conventional one-bug-one-drug paradigm is insufficient to address the challenges of emerging and re-emerging viruses,[5] where medicinal chemistry usually lags behind and develops medication after the pathogen has become a problem. This comes in stark contrast with antibacterial treatments, which are specifically powerful due to the availability of antibiotics with broad antibacterial activity. Such broad-spectrum antiviral agents may have promising prospects for prophylactic or therapeutic settings. For example, several viruses including HIV-1, HSV-2, or ZIKV are transmitted via sexual intercourse,[6,7] and a single broad-spectrum antiviral agent applied topically onto the mucosa would protect against these pathogens. Indeed, condoms that contain a lubricant active against HIV-1 and HSV-2 are already available.[8]

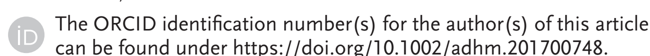
A promising strategy for broad-spectrum antivirals is to block the first step in the viral life cycle—the entry of the virus into the target cell. Most pathogenic viruses are surrounded by a lipid bilayer that harbors viral glycoproteins mediating a first unspecific interaction with ubiquitous cell surface structures, e.g., glycosaminoglycans. Upon engagement of specific receptor molecules, conformational changes in the glycoproteins trigger fusion of viral and cellular membranes, which ultimately leads to infection. Viral adsorption can be modulated by charged polymeric agents. Polycationic macromolecules such as polybrene,[9] protamine sulphate,[10] diethylaminoethyl dextran,[11] or peptide nanofibrils[12] bridge the electrostatic repulsion between negatively charged viral and cellular membranes, thereby increasing rates of virion attachment and infection. This class of positively

F. Schandock, A. Röcker, Dr. J. A. Müller, M. Harms, Prof. F. Kreppel, Prof. J. Münch
Institute of Molecular Virology
Ulm University Medical Center
Meyerhofstrasse 1, 89081 Ulm, Germany
E-mail: Jan.Muench@uni-ulm.de

C. F. Riber, K. B. Løvschall, Dr. A. N. Zelikin
Department of Chemistry
Aarhus University
Aarhus 8000, Denmark
E-mail: zelikin@chem.au.dk

P. Gajda, K. Zuwala, A. H. F. Andersen, Dr. M. Tolstrup
Department of Infectious Diseases
Aarhus University Hospital
Aarhus 8000, Denmark

Dr. A. N. Zelikin
iNano Interdisciplinary Nanoscience Centre
Aarhus University
Aarhus 8000, Denmark


The ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/adhm.201700748.

DOI: 10.1002/adhm.201700748

© 2017 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

charged polymers is widely used as transduction enhancers to boost retroviral gene transfer.[13] A notable exception to this is polyethyleneimine, a cationic polymer which reveals antiviral effects when derivatized with mannose[14] or hydrophobic alkyl side chains.[15] In turn, polyanionic polymers represent the largest class of materials that block viral adsorption and infection. Already in the 1960s, poly-methacrylic acid (PMAA) was shown to suppress the infectivity of enveloped vesicular stomatitis, sindbis, and vaccinia viruses.[16–18] In the past decades, especially with the advent of the acquired immune deficiency syndrome (AIDS) pandemic, several additional polyanionic polymers were described as antivirally active. Among these were sulphated polysaccharides and other sulphated polymers, polyphosphates, carbosilane dendrimers, or nucleic acid polymers, which inhibit a variety of enveloped viruses including HIV-1, HSV-1 and 2, or influenza virus.[19–21] The mechanism underlying virus inhibition is often a direct interaction of the polyanion with viral glycoproteins thereby preventing receptor engagement and fusion.[20,22] Other modes of action are binding of polymers to cellular receptor(s),[20,23] intracellular inhibition of viral polymerases,[23,24] or suppression of viral budding.[25] The broad antiviral activity renders polyanionic polymers interesting candidates for preventive applications. However, a distinct understanding of the structure–activity relationship of this class of antivirals is missing.

In this work, we specifically fill this gap and conduct a systematic variation of polymer structure to understand which aspects of the polymer composition are pivotal for inhibitory antiviral activity against diverse viral pathogens. The specific aim of this study was to identify lead polymers with the broadest spectrum of antiviral activity against major existing, emerging, and re-emerging viral pathogens, and to observe responsible structure–activity characteristics. To achieve this, we synthesized a library of 14 carboxylate, phosphate/phosphonate, and sulfonate polymers differing by anionic functionality and hydrophobicity. These novel polymers were applied against a panel of major viral pathogens, i.e., HIV-1 and HSV-2, as well as pseudoparticles that mimic infection by influenza, Rabies and Lyssa, SARS, Lassa, Marburg, and Ebola viruses. Major focus was set on ZIKV against which currently no drug or vaccine exists. We identified lead candidates with a broad-spectrum but also virus specific antiviral activity. Our results provide a clear suggestive view on what makes a virus susceptible to polymer-based inhibition, and independently, a detailed analysis of which structural characteristics of the polymer make it a superior inhibitor of viral infectivity. We anticipate that the results of this study will prove useful for subsequent development of microbicides with activity against current and re-merging pathogens.

2. Results

2.1. Synthesis of Novel Polyanionic Polymers

Design of polymers with diverse functionalities was performed using a controlled polymerization technique, namely reversible addition fragmentation chain transfer (RAFT) polymerization.[26] This technique is highly tolerant to the presence of functional groups and specifically anions in the polymerization mixture and consequently accommodates a wide variety of monomers. This aspect was deemed pivotal to ensure the synthesis of polymers with the widest possible diversity in terms of chemical composition. We considered polymers with anionic functionalities categorized into (i) carbon-based carboxylates, (ii) phosphor-based phosphates and phosphonates, and (iii) sulfur-based sulfonates. Chemical diversity of commercially available (meth)acrylates and (meth)acrylamides is rather limited with only eight monomers readily available from commercial sources, which led us to design six additional monomers. To our knowledge, two of the phosphate/phosphonate monomers, 2-acrylamidoethyl phosphate (AEP) and 2-acrylamidoethyl phosphonic acid (APA) (corresponding polymers PAEP and PAPA, respectively, **Figure 1**), have never been reported previously.[27] Their methacrylamide counterparts 2-methacrylamidoethyl phosphate (MEP) and 2-methacrylamidoethyl phosphonic acid (MPA) (corresponding polymers PMEP and PMPA, respectively, Figure 1) have been reported previously but not used for controlled polymerization. These monomers are not commercially available and were de novo synthesized from available starting materials. Ethylacrylic acid (EAA) and propylacrylic acid (PAA) are available commercially in small amounts; the monomers were synthesized in one-step syntheses with uncomplicated purifications and good yields, making it possible to obtain large quantities required for polymer synthesis.

Overall, the library of polyanions synthesized herein contained five carboxylate-based polymers, four sulfur-based polymers, and five phosphor-based negatively charged macromolecules (Figure 1). For carboxylates, we were able to accomplish a design strategy that is common in small molecule medicinal chemistry. Thus, the row of polymers from acrylic to methacrylic to ethylacrylic to propylacrylic polyacids is analogous in maintaining polyanionic nature—albeit with an increasing acid dissociation constant—and homologous in a gradually increasing length of the aliphatic side chain, that is, hydrophobicity of the polymer. Addition of hydrophobic groups is a common approach in medicinal chemistry to increase affinity of a small molecule drug to its nominated proteinaceous target.[28] However, this methodology has hardly been applied to probe structure–activity relationship for polymers in biomedicine.

We note that each polymerization required a tedious optimization with regard to the choice of the RAFT agent, the choice of solvent, reaction time, and purification approach. For this reason, resulting polymers were not perfectly matched by molar mass or degree of polymerization and were not devoid of regretful batch-to-batch variation (molar masses and dispersity values are listed in **Table 1** and in the Experimental section). In this work, we therefore focus on the structure–activity relationship with regard to the nature of the anionic charge and hydrophobicity of the polymer. In doing so, we assume that molar mass has an effect secondary to the chemistry of the polymer—as has proven to be the case in a number of recent high throughput optimization studies.[29,30]

2.2. Three Polymers Inhibit ZIKV Infection

Evaluation of antiviral effects exerted by the 14 synthesized polymers was first tested on the viral pathogen that represents

© 2017 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

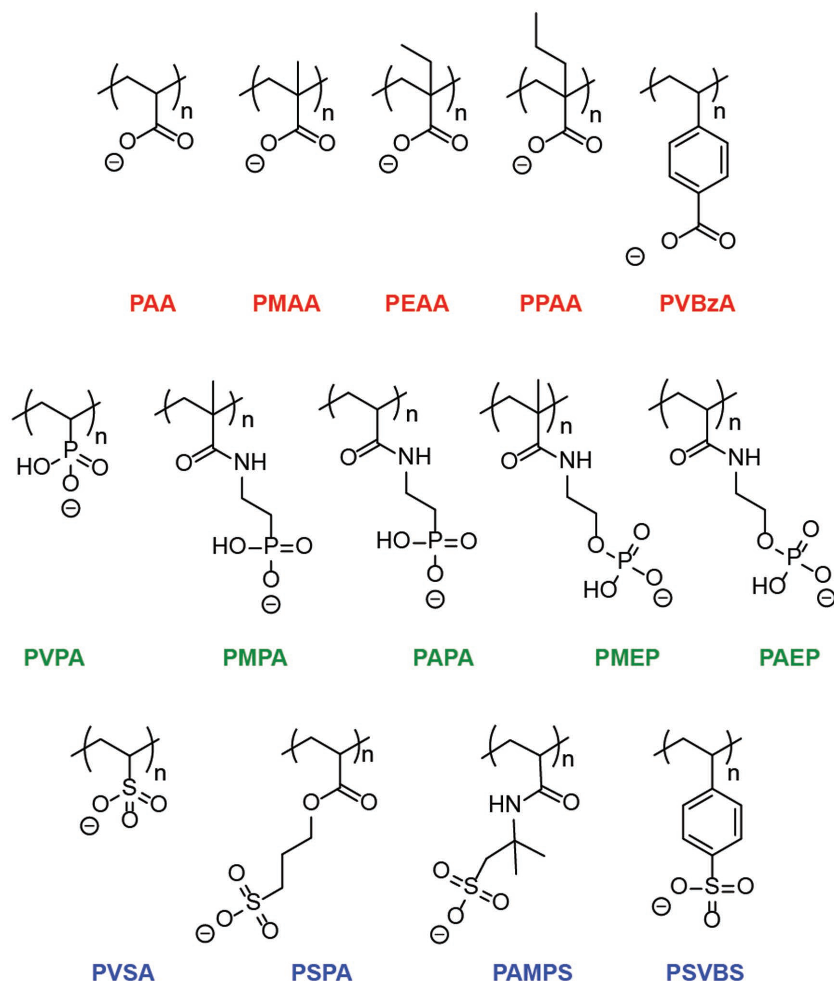


Figure 1. Chemical formulas of the polymers used in this study. Carboxylates (in red): poly(acrylic acid), PAA; poly(methacrylic acid), PMAA; poly(ethylacrylic acid), PEAA; poly(propylacrylic acid); PPAA, poly(vinylbenzoic acid), PVBzA. Phosphates/phosphonates (in green): poly(vinylphosphonic acid), PVPA; poly((2-methacrylamidoethyl)phosphonic acid), PMPA; poly((2-acrylamidoethyl)phosphonic acid), PAPA; poly((2-methacrylamidoethyl)phosphate), PMEP; poly((2-acrylamidoethyl)phosphate), PAEP. Sulfonates (in blue): poly(vinylsulfonic acid), PVSA; poly(3-sulfopropyl acrylate), PSPA; poly(2-acrylamido-2-methyl-1-propanesulfonic acid), PAMPS; poly(vinylbenzenesulfonate), PSVBS.

the most recent viral pandemic, namely ZIKV. We conducted two types of experiments, herein termed “cell treatment” and “virus treatment”. In the “cell treatment” setting, uninfected target cells were incubated with the polymers prior to viral challenge to identify leads that inhibit viral infection and are active in complex cell culture medium containing fetal calf serum. For the “virion treatment” approach, polymers were preincubated with the virus to allow direct interaction between the polymers and then these mixtures were added to target cells resulting in a dilution of the polymer. This approach allows to identify compounds that directly interfere with viral infectivity and may be used as topical microbicides,[31] or in filtration devices for biomedicine and biotechnology to inactivate virus.[32,33]

For the “cell treatment” approach, Vero E6 cells were incubated for 90 min with 0–100 mg L^{-1} of the polymers and infected with the African ZIKV MR766 strain.[34] Infection rates were determined three days later using an 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-based colorimetric assay that quantifies the virally induced cytopathic effect.[35] Results of this screen illustrate, somewhat unexpectedly, that an overall majority of polymers even at their highest concentration are ineffective against ZIKV (**Figure 2**A). However, two polymers, poly(vinylbenzoic acid) (PVBzA) and poly(vinylbenzenesulfonate) (PSVBS), revealed anti-ZIKV activity and shared structural similarity, as these are rather hydrophobic carboxylate- and sulfonate-containing styrenic polymers. These data provide an early indication that the nature of the anionic charge (carboxylate, phosphate/phosphonate, sulfonate) is not decisive in the observed antiviral activity of the polymer. Instead, hydrophobicity of the polymer backbone appears to contribute significantly to the microbicide activity of a polyanion.

Under “virus treatment” conditions, virions were first exposed to 0–100 mg L^{-1} of the polymers for 15 min, and then these mixtures were used to inoculate cells (resulting in a tenfold dilution of the polymers and highest cell culture concentrations of 10 mg L^{-1}). ZIKV infection was quantified as described above through an MTT read-out. This experiment confirmed the anti-ZIKV activity of PVBzA and PSVBS (Figure 2B) and further revealed poly(ethylacrylic acid) (PEAA) as another lead polymer with direct antiviral activity. PEAA has structural similarity with PVBzA and PSVBS, as it also has a hydrophobic backbone. Interestingly, poly(propylacrylic acid) (PPAA) with hydrophobicity of the backbone emphasized by an additional methylene group in each monomer unit as compared to PEAA revealed no antiviral activity. Thus, in a row of carboxylate polymers (PAA, PMAA, PEAA, PPAA) there appears to be an optimized hydrophobic component in PEAA that endows the polymer with enhanced anti-ZIKV activity.

Anti-ZIKV effects exerted by the three lead compounds were further corroborated through quantitative measurements of intracellular levels of the viral E protein using an immunodetection assay.[36] As shown in **Figure 3**A,B, PVBzA and PSVBS inhibited ZIKV MR766 infection under both “cell” and “virion treatment” conditions. The half-maximal inhibitory concentration (IC_{50}) of PSVBS was ≈14 mg L^{-1} in the “cell treatment” assay and 1.4 mg L^{-1} when exposed to virus, demonstrating a direct effect on the virion. PVBzA was markedly less active. PEAA was again only active if pre-exposed to the virus (IC_{50} ≈ 4.1 mg L^{-1}) (Figure 3A,B). Of note, none of the tested polymers were toxic at the concentrations tested (Figure 3C). Thus, out of the 14 polymers studied in this work, three lead compositions suppressed infection of Vero E6 cells with ZIKV.

© 2017 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

Table 1. Macromolecular characteristics (number-average molar mass (M_N) and dispersity ($Đ$)) and 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. For details on polymer characterization, see the Experimental Section and the Supporting Information. Activity of the 14 polymers against pseudoparticles carrying glycoproteins of influenza, Lyssa, Rabies, SARS, Lassa, Marburg, and Ebola viruses.

Polymer	M_N [kDa]	$Đ$	HIV-1		HSV-2	
			IC_{50}, cell treatment [mg L^{-1}]	IC_{50}, virus treatment [mg L^{-1}]	IC_{50}, cell treatment [mg L^{-1}]	IC_{50}, virus treatment [mg L^{-1}]
PAA	28.3	1.1	0.1	0.005	>50	>5
PMAA	57.1	1.2	0.05	0.001	26	0.5
PEAA	4.7	–	23	>5	>50	>5
PPAA	12.6	–	>50	>5	>50	>5
PVBzA	8.7	–	4	0.25	4.8	0.85
PAPA	84.7	1.2	18	>5	>50	>5
PMPA	8.9	1.2	0.7	0.07	>50	>5
PAEP	17.8	1.3	38.5	>5	>50	>5
PMEP	23.4	1.2	0.6	0.03	44	>5
PVPA	12.5	1.3	16.5	>5	14.2	>5
PSPA	37.6	1.4	0.12	0.003	2	0.02
PAMPS	57.2	1.2	0.15	0.002	0.83	0.11
PSVBS	61.4	1.2	0.4	0.04	2.6	0.08
PVSA	1.8	1.2	0.8	0.09	4.3	0.9

To more closely examine the antiviral effect of the lead polymers on an individual cell basis, confocal laser scanning microscopy was performed using the same experimental setup as described above. Upon infection, cells were fixed, and stained for ZIKV E protein, nuclei, and the cytoskeleton (**Figure** 4A). Exposure of cells to 40 and 200 mg L^{-1} PSVBS entirely prevented ZIKV infection (Figure 4A). The staining patterns and intensities of nuclei and cytoskeleton (actin) at 200 mg L^{-1} were unaffected (Figure 4A), confirming lack of cytotoxicity of the polymer. PVBzA also suppressed ZIKV MR766 infection but was less active than PSVBS (Figure 4B, only merged images shown, for controls see Figure S1A of the Supporting Information), confirming the results in Figure 4A,B. Corroboratively, PEAA showed antiviral activity in the virion treatment assay without any sign of cytotoxicity (Figure 4C; Figure S1B of the Supporting Information).

To visualize a possible interaction of polymers with virions, a PEAA-Rhodamine B copolymer was synthesized. As shown in Figure S2A (Supporting Information), the fluorescent polymer blocked ZIKV infection. In the absence of cells and virus, the labeled macromolecule alone did not show fluorescent structures indicating a homogenous distribution of the polymer in solution (Figure 4D). However, in the presence of viral particles fluorescent microscopic aggregates sized 5–20 µm became visible (Figure 4D), which disappeared when the virus inoculum was serially diluted (Figure 4D). Formation of PEAA/ZIKV aggregates was confirmed by flow cytometry using a fluorescein-labeled PEAA (Figure S2B, Supporting Information). These data illustrate coagulation of the viral particles with PEAA and strongly support the notion that inhibitory activity of the polymers is due to direct contact of the macromolecule with the virus.

Experiments so far were performed with the widely used African ZIKV MR766 strain that can be readily analyzed in monkey-derived Vero E6 cells.[34] To more closely mimic physiological conditions, we performed experiments with two clinically relevant ZIKV isolates in a HeLa (human cervical carcinoma) cell line. The isolates are ZIKV FB-GWUH-2016 derived from the brain of an aborted fetus with microcephaly[37] and PRVABC59 obtained from serum of an infected patient from Puerto Rico [obtained from centers for disease control (CDC) and prevention]. As summarized in **Figure 5** and in detail in Figures S3–S5 (Supporting Information), PSVBS and PVBzA ("cell treatment") and PEAA ("virion treatment") almost entirely inhibited infection of HeLa cells by both isolates at concentrations of 40–200 mg L^{-1}. Again, the architecture of the nucleus and the cytoskeleton was unaffected by all three polymers, indicating lack of cytotoxicity and thus specific inhibition of ZIKV infection (Figure 5; Figures S3–S5 of the Supporting Information).

2.3. Effect of the 14 Polymers on HIV-1 Infection

We next investigated if the identified structure–activity relationship is similar for other viral pathogens. We first excluded cytotoxic effects of the polymers which may interfere with interpretation of results derived from the antiviral assays. We tested a panel of mammalian cells used as hosts for the diverse pathogens in this study: HeLa derived TZM-bl for HIV-1, BHK (ELVIS) cells for HSV-2, hepatic Huh-7, HEK293T, and Vero E6 cells for remaining viruses. Toxicity of polyanions was quantified in these cells up to 50 mg L^{-1} concentration of the polymers (TZM-bl, Huh7, ELVIS, and HEK293T), or 200 mg L^{-1} for Vero E6 cells, being the highest concentration of polymers used in respective experiments. Toxicity screening by MTT assay revealed that decrease in metabolic activity of the cells upon incubation with polymers was marginal (Figure S6, Supporting

© 2017 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

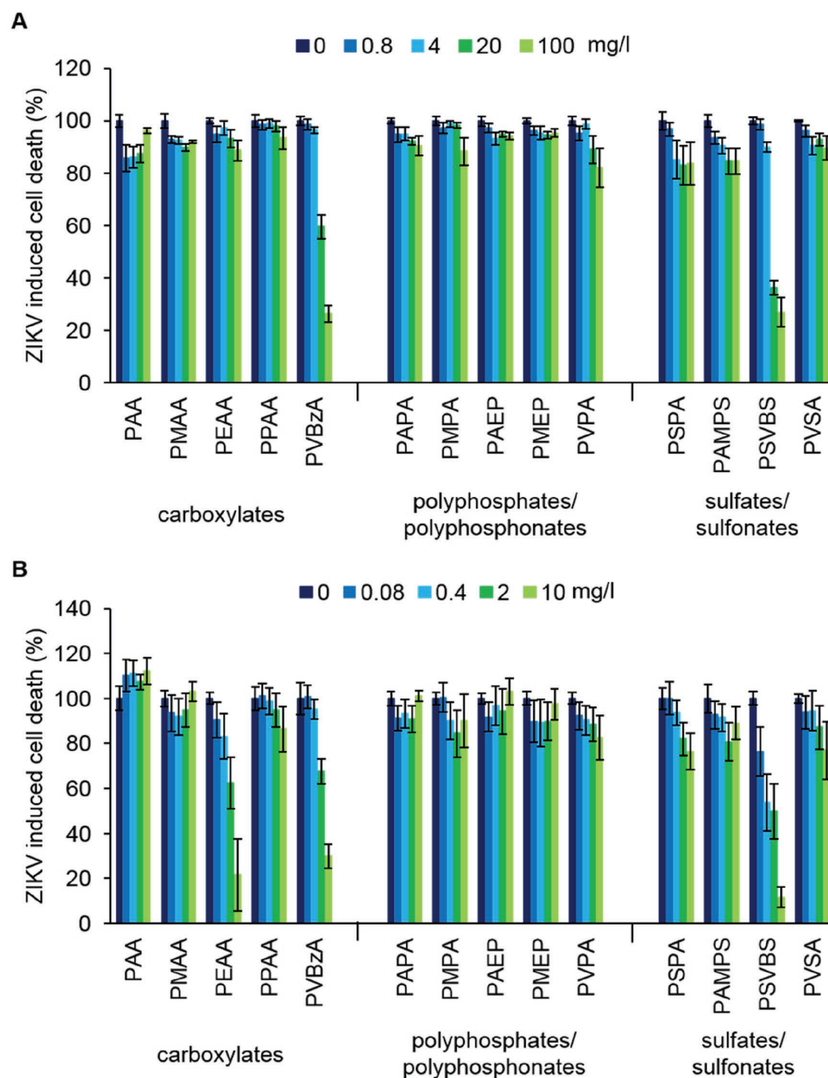


Figure 2. Identification of polyanionic inhibitors of ZIKV. A) Vero E6 cells were incubated with 0–100 mg L^{-1} of the polyanions for 90 min and then infected with ZIKV MR766 ("cell treatment"). Infection rates were determined 3 d later by MTT assay. B) ZIKV MR766 was incubated for 15 min with polyanions (0–100 mg L^{-1}) and then mixtures were added to Vero E6 cells, resulting in tenfold dilutions of the potential drugs, as indicated in the legend ("virion treatment"). Results are presented as mean ± standard error derived from two independent experiments performed in triplicate for each data point.

Information) and cannot account for antiviral effects described below.

Inhibitory activity of the polymers was tested on HIV-1 infection using TZM-bl cells that express β-galactosidase upon viral infection.[38] In stark contrast to data obtained with ZIKV (Figure 2), most polyanions suppressed HIV-1 infection to a level below 20% under both cell and virus treatment conditions (**Figure 6**; Figure S7 of the Supporting Information). IC_{50} values indicate that similar to ZIKV, "virus treatment" was typically superior to "cell treatment", which is most prominent for poly(3-sulfopropyl acrylate) (PSPA) and poly(2-acrylamido-2-methyl-1-propanesulfonic acid) (PAMPS) (Table 1). This observation reiterates that for polyanions, antiviral activity of the polymer is characterized with higher potency if the polymer is allowed to come into contact with the viral particles prior to being administered onto cells. For HIV-1, it appears that "strength" of the polyanions as known from polyelectrolyte complexation[39] is decisive and that the sulfur-containing polymers are most potent, with IC_{50} values for "virus treatment" being on the order of only a few μg L^{-1} (Table 1). Of note, other polyanionic polymers such as heparin, dextran sulfate, polyphosphates, or carbosilane dendrimers are less potent HIV-1 inhibitors with IC_{50} values in the high μg L^{-1} to low mg L^{-1} range.[19,21,31,40,41] On par with the polysulfonates, carboxylates PAA and especially PMAA were also highly potent. By contrast, PEAA and PPAA, two polymers with significantly higher p*K*a (pK_a 6.5 vs 4.5 for PAA) and hence markedly lower ionization at physiological pH, were significantly less active and had IC_{50} values >5 mg L^{-1}. Moreover, the three hydrophobic leads identified for the ZIKV treatment are rather inferior inhibitors in the case of HIV-1. Therefore, the hydrophobicity of polymers does not appear to be a strong contributing factor to the overall inhibitory effect of the polymer as is the case for ZIKV.

2.4. Effect of the 14 Polymers on HSV-2 Infection

Antiviral activity of polyanions against HSV-2 was tested in ELVIS cells that contain an HSV inducible *β*-galactosidase gene, which is expressed upon infection via the viral transactivator ICP10.[42] Reporter enzyme activities were quantified 36 h post infection before a virally induced cytopathic effect occurred and are shown in **Figure 7** ("virus treatment") and Figure S8 of the Supporting Information ("cell treatment"). As with HIV-1 experiments, exposure of the virus to the polymer prior to administration to cells resulted in a higher antiviral effect as the IC_{50} values in "virus treatment" were 10–100-fold lower than those for the "cell treatment" (Table 1). Polysulfonates revealed potency as low as 0.01 mg L^{-1} for PSPA in the "virus treatment" setup and sub 1 mg L^{-1} potency for PAMPS in a "cell treatment" setup. The most potent carboxylates had IC_{50} values of 0.5 mg L^{-1} for PMAA for the "virus treatment," although this value was nearly 200-fold higher in the "cell treatment" approach. Phosphor-based polymers had IC_{50} values exceeding the range of concentrations tested in this experiment.

These results were fully corroborated in a plaque reduction assay which allows assessing effects of antivirals on spreading viral infection (Figures S9 and S10, Supporting Information). Viral proliferation within confluent cultures of

© 2017 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

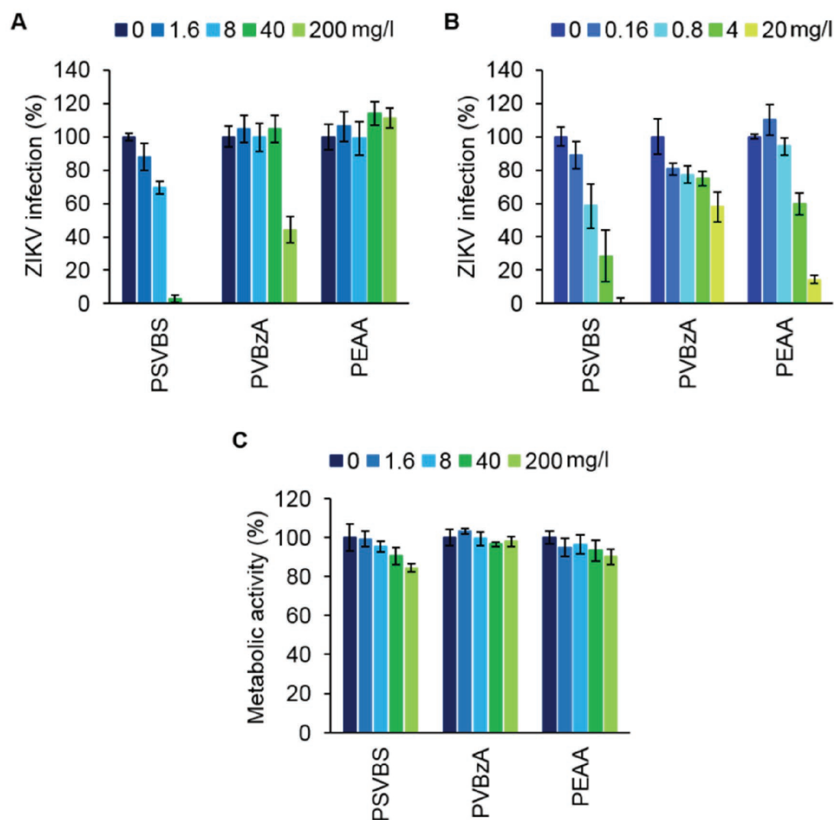


Figure 3. Lead polymers inhibit ZIKV. A) PSVBS, PVBzA but not PEAA inhibit ZIKV MR766 infection of Vero E6 cells under "cell treatment" conditions. The experiment was performed as described in Figure 2A but infection was quantified 36 h post infection by virus immunodetection assay using the flavivirus antibody 4G2. B) PSVBS, PVBzA, and PEAA block ZIKV MR766 infection of Vero E6 cells under "virion treatment" conditions, as measured by ZIKV immunodetection assay. C) The three anti-ZIKV polymers are not toxic. Vero E6 cells were exposed to the three polymers and cell viability was assessed by MTT 3 d later. Results are presented as mean ± standard error derived from two independent experiments performed in triplicate for each data point.

Vero E6 cells leads to formation of well-visible virus induced plaques, whereas inhibition of the viral proliferation affords a picture devoid of these plaques. Confirming the finding listed above, we observed that polyanions differed greatly in their antiviral activity. The sulfur-based polyanions proved to be highly potent inhibitors of HSV-2. By contrast, phosphor-based polymers revealed minor, rather insignificant activity against HSV-2. Among the carboxylates, the polymer with the highest anionic character, PAA, exhibited virtually no anti-HSV-2 activity. Benzylic PVBzA and methacrylic PMAA proved to be the strongest inhibitors of HSV-2 among these polymers.

We next determined the effect of the 14 polymers against influenza (H5N1) and a panel of viruses for which currently no antiviral therapy exists. For this, we generated lentiviral pseudoparticles encoding luciferase that were pseudotyped with the glycoproteins of Lyssa and Rabies virus (two neurotropic rhabdoviruses that cause rabies in humans and animals), Ebola and Marburg virus (which belong to the family of filoviruses and are responsible for sporadic outbreaks of fatal hemorrhagic fevers in Africa), SARS coronavirus (that caused the SARS epidemic in 2002), and Lassa virus (an arenavirus that causes Lassa fever). In addition, polymers were analyzed for inhibition of human adenovirus type 5, a nonenveloped virus with clinical relevance for immunosuppressed (transplant) patients.[43,44] As shown in **Figure 8** and Figure S11 (Supporting Information), none of the polymers led to marked inhibition of adenovirus infection, whereas all tested pseudoparticles with the exception of influenza were inhibited by at least two polymers by more than 80% (For overall results of inhibitory activity of the polymer, see **Figure 9**). An important observation from this screen is that "standard" strong polyelectrolytes such as PAMPS or highly ionized PAA do not reveal broadness of antiviral activity, and that negative charge alone does not make the polymer a strong antiviral agent. This is further illustrated by the inhibitory activity of poly(phosphates/phosphonates): for all the viral particles, inhibitory effects of these polymers were minimal to nonexistent. A surprising exception to this conclusion is poly(vinylphosphonic acid) (PVPA) which proved to be highly effective against Marburg, SARS, and influenza virus, particularly for SARS (Figure 9). For influenza, PVPA proved to be the most effective of all the polyanions tested in this work. Among the sulfur-containing polymers, styrenic PSVBS appears to be the strongest lead being the most effective inhibitor in this range of polymers for each of the pseudotyped viral particles (Figure 8). Similarly, PVBzA appears to be the strongest lead among the carboxylates in which case hydrophobic PEAA is also a good lead being particularly effective against the filoviruses (Ebola and Marburg). Taken together, the data in this screen strongly support the notion that combination of anion charge and hydrophobicity of the polymer backbone is the necessary combination that makes up effective broadly acting antiviral polymers.

3. Discussion

In this work we implemented the methodology of classic medicinal chemistry (systematic variation and extension of structure) to design macromolecular drugs against major viral pathogens. We show that for each enveloped virus at least one polymer can be identified with antiviral activity—illustrating that polymer-assisted inhibition of viral infectivity is a rather universal phenomenon. Specifically, our data suggest that hydrophobicity of the polymer backbone is pivotal to broaden antiviral activity of the anionic macromolecule. The overall lead of this study, poly(vinylbenzoic acid), PVBzA, suppressed infectivity of all the enveloped viruses studied in this work and may have prospects for further development as broad-spectrum preventative antiviral agent.

© 2017 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

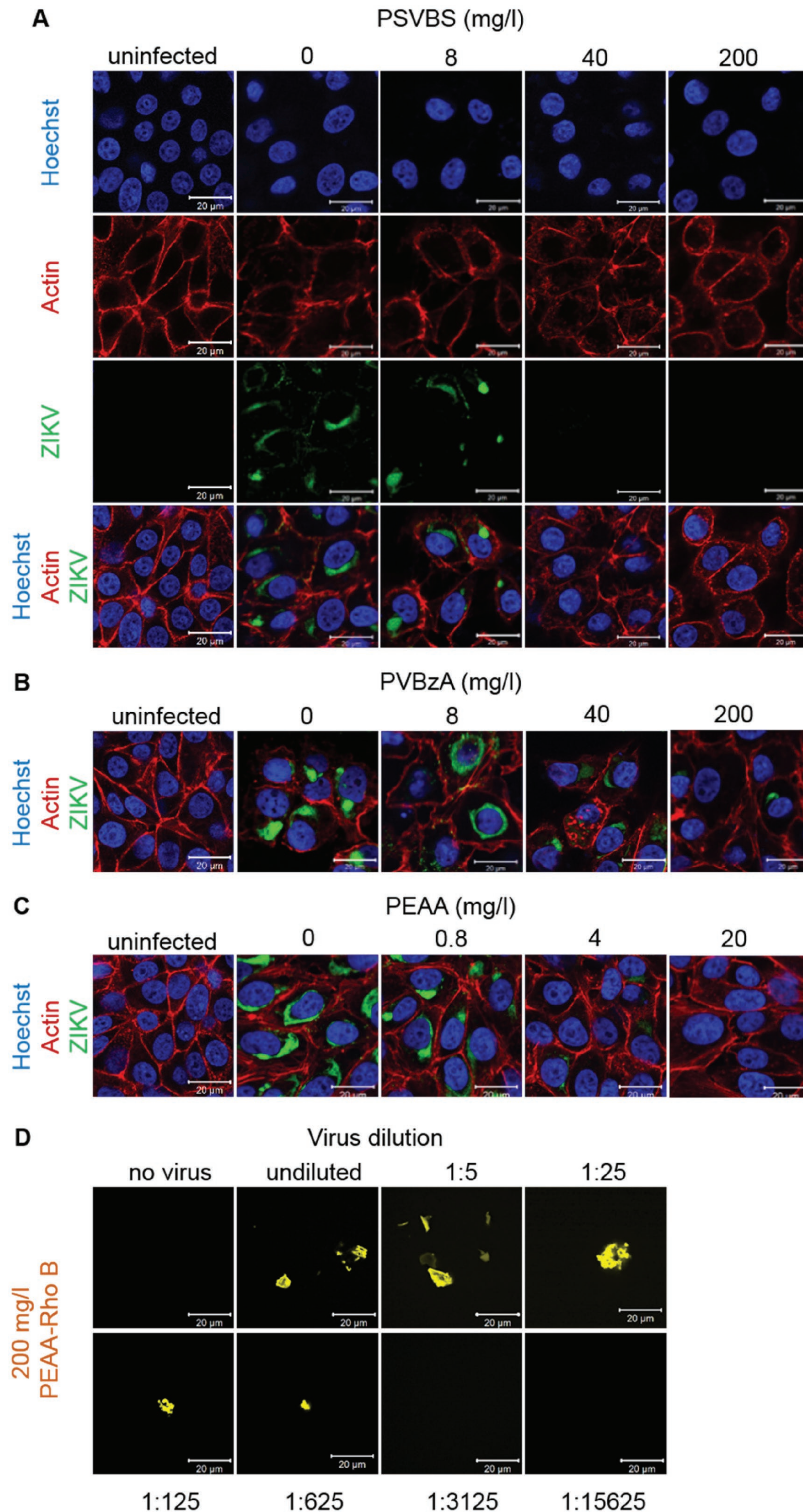


Figure 4. Fluorescence microscopy of ZIKV inhibition by polymers. A) PSVBS and B) PVBzA inhibit Vero E6 cell infection under "cell treatment" conditions (also see Figure S1A, Supporting Information). C) PEAA blocks ZIKV when pre-exposed to virions (also see Figure S1B, Supporting Information). To visualize infection, cells were fixed, incubated with the anti-ZIKV mouse antibody 4G2 that detects the viral E glycoprotein, and labeled with a secondary goat anti-mouse IgG coupled to Alexa Flour 488 (green). Cell nuclei were stained with Hoechst (blue), and the cytoskeleton with labeled actin-specific phalloidin (red). Shown are merged confocal images taken 3 d post infection. D) Rhodamine B-coupled PEAA interacts with ZIKV. Rhodamine B-coupled PEAA (200 mg L^{-1}) was incubated with buffer (no virus) or indicated dilutions of the ZIKV MR766 stock for 15 min and then imaged by confocal microscopy in the absence of cells (see Figure S2, Supporting Information).

3.1. PEAA, PVBzA, and PSVBS are Potent Inhibitors of ZIKV Infection

Specific novelty of this work lies in that we consider polyanions as antiviral agents against ZIKV and other (re)-emerging pathogens. Our in-depth analysis of the polymers for anti-ZIKV activity identified two carboxylates, PEAA and PVBzA, and the polysulfonate, PSVBS. The latter was most potent and inhibited clinically relevant ZIKV strains in physiologically relevant cell types with IC_{50} values in the mg L^{-1} range without causing any cytotoxic effects. This comes in contrast with recent reports on the two prototype polyanions, dextran sulfate and heparin, which were shown to exert only marginal antiviral activity against ZIKV.[45,46] For the carboxylate PEAA we provide evidence for a direct interaction with the virion, demonstrating that the identified lead polymers inhibit ZIKV with different mechanisms. A promising ad hoc application of polymers is their use as topical microbicide. It is now well established that besides mosquitos, ZIKV can also be transmitted via sexual intercourse.[6,7] Sexual transmission also results in ZIKV fever[6,7] and potentially microcephaly and brain malformations in fetuses.[47,48] Vaginally applied microbicides may help to protect woman at risk for acquiring ZIKV and other sexually transmitted viruses. Thus, animal studies to analyze the effect of PEAA, PVBzA, and PSVBS for prevention of vaginal ZIKV infection in mice models are highly warranted.

3.2. Susceptibility of Viruses to Polyanionic Polymers

From the standpoint of structure–activity relationship for viruses, our data suggest that inhibition by polymers is related to the presence and the density of viral glycoproteins embedded into the viral envelope. Indeed, nonenveloped AdV appears to be least susceptible to polymeric inhibitors, which is in good agreement with prior observations

© 2017 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

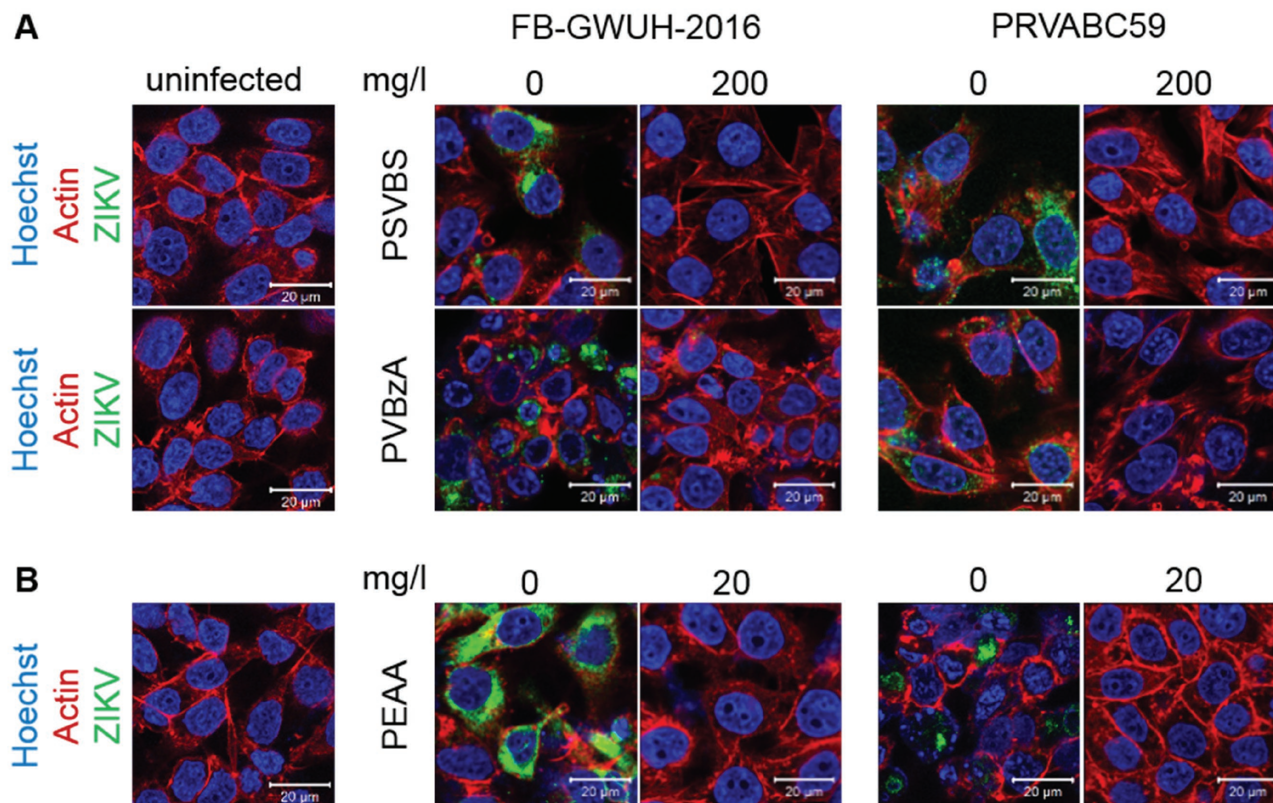


Figure 5. Polymers inhibit clinically relevant ZIKV isolates. A) HeLa cells were exposed to PSVBS and PVBzA for 90 min and then infected with the FB-GWUH-2016 and PRVABC59 ZIKV isolates. B) Both viruses were exposed to PEAA for 15 min, and then used to infect HeLa cells. Three days later, cells were fixed, stained for ZIKV E protein (green), nuclei (blue), and actin (red), and analyzed by confocal microscopy (also see Figures S3 and S4, Supporting Information).

on this subject.[19] The ZIKV surface is densely packed with GPs[49,50] and is only inhibited by few lead polymers. In turn, HIV-1 is unique in that it incorporates only a few GPs[51,52] and activity of polymers against HIV-1 is almost trivial and observed for nearly all polymeric inhibitors, here and in numerous inhibitors in prior art.[18,19,53] The HSV-2 surface is intermediate in terms of coverage with GPs[46] and indeed this virus is inhibited by a greater number of polymers in our screen as compared to ZIKV but fewer than HIV-1. Our data indicate that lack of the envelope or an envelope with a dense GP shield makes viruses less susceptible to inhibition by the polymers. By contrast, sparse

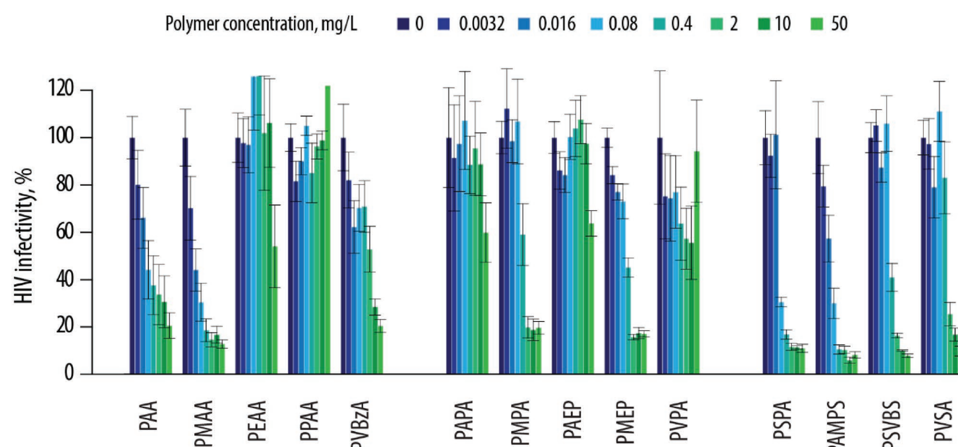


Figure 6. Inhibitory activity of polyanions against HIV-1 ("virus treatment" mode). Polymers were first mixed with HIV-1 for 10 min and then these mixtures were used to inoculate TZM-bl cells. Infection rates were determined 3 d later by quantifying β-galactosidase activities in cellular lysates. Results are presented as mean ± standard error derived from two independent experiments performed in triplicate for each data point.

© 2017 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

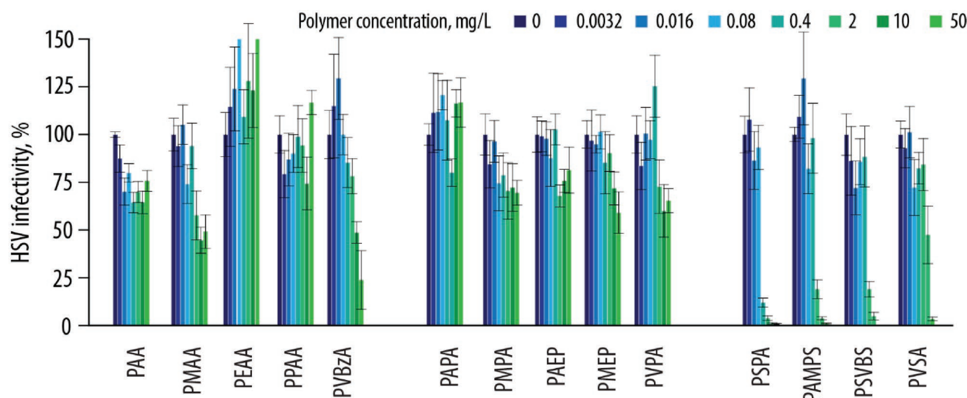


Figure 7. Inhibitory activity of polyanions on infectivity of HSV-2 under "virus treatment." HSV-2 particles were exposed to polymers, incubated for 1 h, and the mixtures were used to inoculate ELVIS cells. Infection rates were determined 36 h later through quantifying β-galactosidase activities in a chemiluminescence-based assay. Results are presented as mean ± standard error derived from two independent experiments performed in triplicate for each data point. For results obtained in the "cell treatment" setup, see Figure S8 (Supporting Information).

or moderate coverage of the enveloped viral particle makes the virus well susceptible to the polymer-mediated inhibition, likely due to the more or less prominent interaction with the different viral GPs[18,54–56] and plausibly interaction with the lipid bilayer.[15]

3.3. Structure–Activity Relationship for the Polymers

From the standpoint of structure–activity relationship for the polymers, the first important conclusion from this work is that polysulfonates, strong polyanions which are historically deemed the prime microbicide candidates,[18,53,57] do not make up broad spectrum antiviral materials. Specifically, polymers in this family did not reveal notable inhibitory activity on influenza, SARS, or (except for PSVBS) against ZIKV. Another significant surprise is the spectrum of activity for the vinylic PVPA. Polyphosphates/phosphonates proved to be virtually devoid of any antiviral activity in our screens but PVPA was a notable exception. This polymer scored well against nearly all pathogens except for ZIKV and Ebola, and revealed highest activity against HSV-2 and SARS. Finally, the very noticeable finding of this study is the overall "lead" identified herein, namely PVBzA, a polycarboxylate with a hydrophobic backbone. This polymer exhibited superior (compared to other polymers) activity against each virus used in this work except for the nonenveloped AdV. Thus, a combination of anionic charge and hydrophobicity broadens the antiviral activity of a polymer and PVBzA represents a highly promising candidate for further preclinical studies and microbicide development.

3.4. Conclusions and Outlook

In this work, we performed a screen of 14 polymers as inhibitory agents against 11 viral pathogens and provide a clear suggestive view on what makes a virus susceptible to polymer-based inhibition and independently, a detailed analysis of which structural characteristics of the polymer make it a superior broad inhibitor of viral infectivity. We show that for enveloped viruses, at least one of the polymers can be found to have an antiviral effect—illustrating that polymer-assisted inhibition of viral infectivity is a rather universal phenomenon. Specific novelty of this work lies in our consideration of polyanions as antiviral agents against ZIKV, SARS, Ebola, and other pathogens that caused very recent pandemics. Further novelty is in that we implement the methodology of classic medicinal chemistry (systematic variation and extension of structure) into the design of macromolecular drugs. We believe that this aspect allowed us to identify the leads that are rather unexpected when considered in the overall context of macromolecular antiviral agents. Specifically, our data strongly suggest that hydrophobicity of the polymer backbone is pivotal for antiviral activity of the macromolecule. The overall lead of this study, poly(vinylbenzoic acid), PVBzA, suppressed infectivity of all the enveloped viruses studied in this work. Identified leads are promising candidates for microbicides and could be formulated as antiviral crèmes, gels, sprays, paints, and coatings for diverse biomedical and biotechnological applications.[8–10] Future work with the identified lead polymer(s) will focus on three aspects: We will clarify whether identified anti-ZIKV leads prevent ZIKV infection via vaginal challenge in mice models to evaluate their potential for microbicide development. We will clarify in detail the mechanism of antiviral activity of polymers and identify if the viral envelope, the glycoproteins, or both is the cause of the essential polymer–virus contact. And, we aim to optimize the antiviral effects of the lead polymers to achieve higher efficacy of the antiviral treatment, specifically through variation of the polymer architecture—linear polymer, branched, hyperbranched or dendritic backbone, star polymers.

4. Experimental Section

Unless stated otherwise, all reagents were purchased from Sigma-Aldrich and used without purification.

Monomer Synthesis: MPA, APA, MEP, AEP, EAA, and PAA were synthesized as described in detail in the Supporting Information.

Polymer Synthesis: All polymers were synthesized via RAFT polymerization technique using azobisisobutyronitrile (AIBN), 2,2′-azobis(4-methoxy-2.4-dimethyl valeronitrile) (V-70) or

© 2017 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

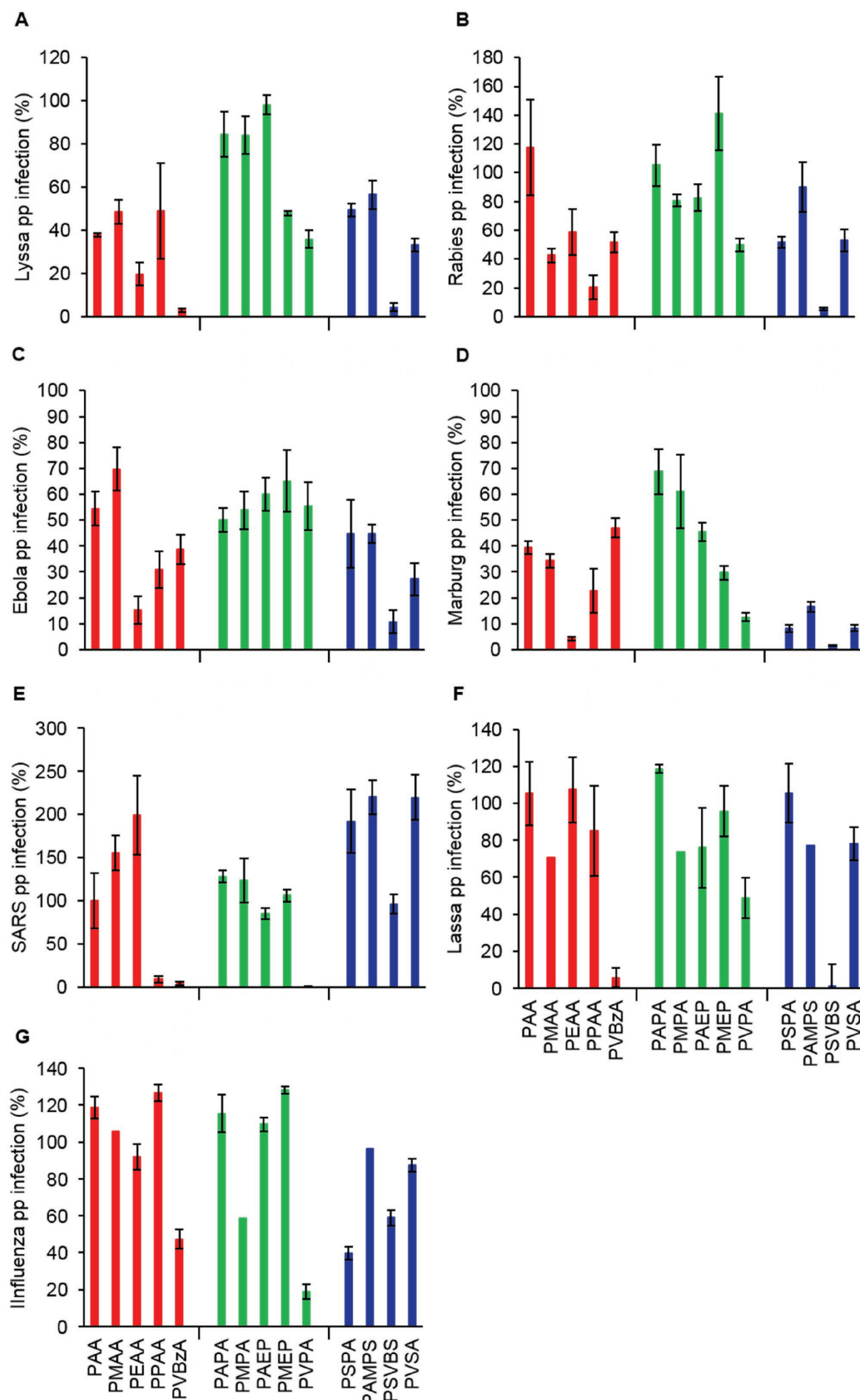


Figure 8. Antiviral activity of polyanions against lentiviral pseudotypes carrying glycoproteins derived from A) Lyssa virus, B) Rabies virus, C) Ebola virus, D) Marburg virus, E) SARS virus, F) Lassa fever virus, G) influenza virus. Respective target cells were exposed to 50 mg L^{-1} of the polymers and then infected with the pseudoparticles. Cellular entry is mediated by the glycoproteins and infection rates were determined 3 d post infection by quantifying luciferase activities in cellular lysates. Shown are the mean % infection rates obtained from polyanion treated cells relative to the untreated control (100%). Results are presented as mean ± standard error derived from two independent experiments performed in triplicate for each data point. pp: pseudoparticle.

© 2017 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

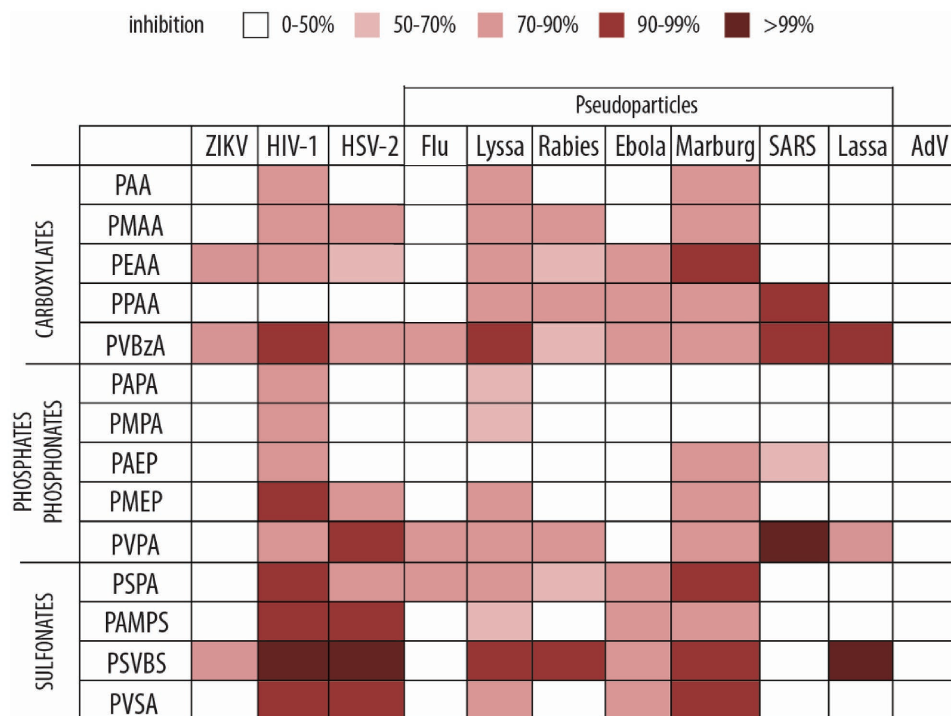


Figure 9. Summary of the maximum antiviral activities of tested polyanions.

4,4′-azobis(4-cyanovaleric acid) (ACVA) as an initiator and cyanomethyl dodecyl trithiocarbonate (CDTC), (4-cyano-4-[(dodecylsulfanylthiocarbonyl)-sulfanyl]pentanoic acid) (CDPA), cyanomethyl methyl(phenyl)-carbamodithioate (CMPD), 2-(dodecylthiocarbonothioylthio)-2-methylpropionic acid (DCMA), or 4-cyano-4-(phenylcarbonothioylthio)-pentanoic acid (CPPA) as a RAFT agent in dimethylformamide as a solvent, as described in detail in the Supporting Information. The following initiator + RAFT agent combinations were used for the syntheses of polymers: PAA: AIBN + CDTC; PMAA: AIBN + CDPD; PEAA: V-70 + CDPA; PPAA: V-70 + CDPA; PVBZ: AIBN + CMDP; PVSA: AIBN + CMPD; PSPA: AIBN + DCMA; PAMPS: AIBN + DCMA; PSVBS: AIBN + DCMA; PVPA: V-70 + CMPD; PMPA: ACVA + CPPA; PAPA: V-70 + CDPA; PMEP: V-70 + CDPA; PAEP: V-70 + CDPA.

Polymer Analysis: Size-exclusion chromatography was performed using a system comprising a LC-20AD Shimadzu HPLC pump, a Shimadzu RID-10A refractive index detector, and a DAWN HELEOS 8 light scattering detector along with a SPD-M20A PDA detector, equipped with either (1) a HEMA-Bio Linear column with 10 µm particles, a length of 300 mm, and an internal diameter of 8 mm from MZ-Analysentechnik in series with a OHpak SB-803 HQ Shodex column with the dimensions 8.0 × 300 mm a particle size of 6 µm or (b) Mz-Gel SDplus Linear column with 5 µm particles length of 300 mm and an internal diameter of 8 mm from MZ-Analysentechnik providing an effective molecular weight range of 1000–1 000 000. The solvent used was either (a) 0.01 M PBS filtered through a 0.1 µm filter with 300 ppm sodium azide or (b) dimethylformamide (DMF) with 10×10^{-3} M LiBr added. The d*n*/d*c* used to calculate the molecular weights of the polymers was determined by assuming full mass recovery. Results of polymer characterization are presented in the Supporting Information.

Cell Viability: To access cell viability, all applied cell lines (10 000 TZM-bl cells, 5000 ELVIS cells, 10 000 HEK293T cells, 10 000 Huh7 cells, or 6000 Vero E6 cells) were seeded into 96-well plates and incubated with the highest polymer concentrations used in the respective assays. After 3 d of incubation, the medium was discarded and 100 µL of 0.5 mg mL^{-1} MTT-PBS (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide) solution was added to the cells. Live cells reduce the yellow MTT salt by NAD(P)H-dependent oxidoreductase system causing the formation of insoluble purple formazan crystals. After 2 h, the cell-free supernatant was discarded and formazan crystals were dissolved in 100 µL dimethylsulfoxide (DMSO):Ethanol (1:2). Absorption was detected at 490 nm and corrected by the background absorption at 650 nm.

Effect of Polymers on ZIKV Infection: The initial screen was performed by MTT-based colorimetric detection assay:[35] Vero E6 cells were cultured in Dulbecco's modified essential medium (DMEM) supplemented with 2.5% (v/v) heat-inactivated fetal calf serum (FCS), 2×10^{-3} M L-glutamine, 1×10^{-3} M sodium pyruvate, 1× nonessential amino acid (Sigma), 100 units mL^{-1} penicillin, and 100 µg mL^{-1} streptomycin. 6000 Vero E6 cell were seeded into 96 well plates. The next day, 0–100 µg mL^{-1} of the polymers were added in triplicates to the cells ("cell treatment") and incubated for 90 min at 37 °C before the cells were infected with ZIKV strain MR766, a ZIKV strain that was isolated in 1947 from a sentinel rhesus macaque.[34] In the "virus treatment" setting the polymers (0–100 mg L^{-1}) were preincubated with the virus ZIKV MR766 for 15 min at 37 °C before using these mixtures to infect the cells in triplicates. 3 d post infection MTT was added to the cells. Formazan crystals formed by live cells were measured as described above, and used to indirectly detect ZIKV induced cell death and to assess protection by polyanions.

Effective polymers were reassessed (in concentrations up to 200 µg mL^{-1}) in a cell-based ZIKV immunodetection assay. The "cell" and "virus treatment" experiments were performed as described. 36 h post infection, cells were rinsed with PBS, fixed for 20 min at room temperature with 4% paraformaldehyde, permeabilized with cold methanol for 5 min at 4 °C, and washed with PBS. Next, cells were incubated with mouse antiflavivirus antibodies 4G2 in PBS containing 10% (v/v) FCS and 0.3% (v/v) Tween20 for 1 h at 37 °C. Following times times of washing with PBS containing 0.3% (v/v) Tween20, cells were incubated with a horseradish peroxidase (HRP)-coupled anti-mouse antibody (1:20 000) for 1 h at 37 °C. Next, cells were washed four times and tetramethylbenzidine (TMB) substrate was added. After 5 min 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.

© 2017 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

To visualize ZIKV infection, laser scanning microscopy was performed. 24 000 Vero E6 cells were seeded into an 8-well Ibidi slide. The next day, cells were incubated with PSVBS and PVBzA and infected after 90 min incubation at 37 °C with ZIKV MR766 ("cell treatment"). For "virus treatment," ZIKV MR766 was incubated with PEAA and fluorescent PEAA Rhodamine B copolymer (PEAA-Rho B) for 15 min and the mixture was used to infect Vero E6. For both treatments, 3 d post infection the cells were fixed for 10 min at 4 °C with 4% paraformaldehyde and permeabilized with 0.1% Triton-X-100. Afterward, a blocking solution containing 5% (v/v) FCS and 1% (v/v) bovine serum albumin (BSA) was used to incubate the cells for 30 min at room temperature. To visualize the ZIKV infection, mouse antiflavivirus antibodies 4G2 (1:100 (v/v)) and secondary Alexa Flour 488 goat anti-mouse IgG (molecular probes) (1:1000 (v/v)) were used. Both antibodies were in solution with phosphate buffered saline (PBS) containing 1% (v/v) BSA and incubated with the cells for 45 min at room temperature. Next, nucleus was stained with Hoechst 33342 (ThermoFisher Scientific) (1:2000 (v/v)) and afterward actin filaments were stained with Phalloidin-Atto 647N (Attotec) (1:400 (v/v)). Cells were stored in PBS, kept in the dark and left at 4 °C until microscopy image acquisition was performed using a Zeiss LSM 710 and ZEN software 2010 for image processing. To analyze interaction of ZIKV with PEAA, different ZIKV MR766 amounts were incubated with 200 μg mL^{-1} PEAA-Rho B in an 8-well Ibidi slide for 15 min. Afterward the mixture was fixed with 4% paraformaldehyde and microscopy image acquisition performed. Antiviral activity of polyanions against clinical ZIKV isolates (ZIKV FB-GWUH-2016 (ZIKV FB) derived from the brain of an aborted foetus with microcephaly[37] and PRVABC59 (PRVABC) obtained from serum of an infected patient from Puerto Rico [obtained from CDC] was assessed in 30 000 HeLa cells cultured in DMEM supplemented with 100 units mL^{-1} penicillin, 120 μg mL^{-1} streptomycin, 2×10^{-3} M L-glutamine, 1× nonessential amino acids, and 10% (v/v) FCS.

Effect of Polymers on HIV-1 Infection: Virus stocks of CCR5-tropic HIV-1 NL4-3 92TH014.12[58] were generated by transient transfection of HEK293T cells.[59] After transfection and overnight incubation, the transfection mixture was replaced with 2 mL DMEM with 2.5% inactivated FCS. After 40 h, the culture supernatant was collected and centrifuged for 3 min at 330 × g to remove cell debris. Virus stocks were stored at –80 °C. The reporter cell line TZM-bl was obtained through the NIH ARRRP and cultured in DMEM supplemented with 100 units mL^{-1} penicillin, 120 μg mL^{-1} streptomycin, 2×10^{-3} M L-glutamine, and 10% FCS. 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.

In the "virus treatment" assay, HIV-1 particles were incubated with polymers (0–50 mg L^{-1}) for 10 min at 37 °C. Then, these mixtures were used for triplicate infection of 10 000 TZM-bl cells seeded the day before into 96-well plates. For the "cell treatment" assay, 0–50 μg mL^{-1} of the polymers were added in triplicates to the cell culture medium and incubated for 1 h at 37 °C before the cells were infected. HIV-1 infection rates were determined 3 d post infection by detecting β-galactosidase activity in cellular lysates using the Gal-Screen β-Galactosidase Reporter Gene Assay System for Mammalian Cells (Thermo Fisher Scientific) and the Orion II microplate luminometer (Berthold, Bad Wildbad, Germany). Reporter gene activities (recorded as relative light units per second) obtained from infected cells containing no polyanion were used as control (100%).

Effect of Polymers on HSV-2 Infection Using ELVIS Reporter Cells: Virus stocks of HSV-2 green fluorescent protein (GFP) were generated by infecting ELVIS cells (genetically engineered baby hamster kidney cells that express β-galactosidase upon infection with HSV-2).[42] After 48 h, the culture supernatant containing progeny virus was collected and centrifuged for 3 min at 330× g to remove cell debris. Virus stocks were stored at –80 °C. ELVIS cells were cultured in DMEM supplemented with 100 units mL^{-1} penicillin, 120 μg mL^{-1} streptomycin, 2×10^{-3} M glutamine, and 10% FCS. The DNA bears an HSV inducible promoter gene which 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. This was used to determine infection rates 36 h post infection by detecting the β-galactosidase activity in cellular lysates using the Gal-Screen β-Galactosidase Reporter Gene Assay System for Mammalian Cells (Thermo Fisher Scientific) and the Orion II microplate luminometer (Berthold, Bad Wildbad, Germany). All values represent reporter gene activities (relative light units per second; RLU s^{-1}) derived from triplicate infections minus background activities derived from uninfected cells. Shown are percent infection rates relative to the untreated infected control (100%).

In the "virus treatment" assay, HSV-2 was incubated with polymers (0–50 mg L^{-1}) for 10 min at 37 °C. Then, these mixtures were used for triplicate infection of 5000 ELVIS cells seeded the day before into 96-well plates. For the cell treatment assay, 0–50 mg L^{-1} of the polymers were added in triplicates to the cell culture medium and incubated for 1 h at 37 °C before the cells were infected with HSV-2.

Effect of Polymers on HSV-2 Infection Using Vero Cells: HSV-2 strain 333 was produced by infecting Vero cells and harvesting supernatant 48 h later. The supernatant was centrifuged at 300 g for 5 min. Virus stocks were stored at –80 °C. Vero cells were cultured in DMEM (Lonza, Basel, Switzerland) containing 10% heat-inactivated FCS and 50 U mL^{-1} penicillin and 50 μg mL^{-1} streptomycin (Invitrogen, Glostrup, Denmark). The inhibition of HSV-2 was determined through "virus treatment" assay. The cells were seeded on 24-well plate at the density of 2 × 105 cells per well and cultured overnight. After overnight culture the media was replaced with fresh one. The virus (titer: 1000 pfu mL^{-1}) was incubated with the drugs at initial concentrations of 500 and 50 mg L^{-1} for 30 min at the bench and then added to the cells. The final concentrations of the drugs were 100 and 10 mg L^{-1}, and viral titer was 200 pfu mL^{-1}. 24 h later a standard plaque assay was performed. Briefly, media was removed and the cells were fixed in 4% paraformaldehyde (PFA) solution for 10 min. Subsequently the cells were washed twice with PBS. 0.5% solution of crystal violet in PBS containing 10% ethanol was used to stain the cells for 10 min. The stain was washed away with PBS. Plaques were enumerated under the microscope. The number of plaques was normalized to the control sample infected with HSV-2 and presented as a percentage of the control.

Effect of Polymers on Pseudoparticles: HEK293T cells were cultured in DMEM supplemented with 100 units mL^{-1} penicillin, 120 μg mL^{-1} streptomycin, 2×10^{-3} M L-glutamine, and 10% FCS. Huh7 cells were kindly provided by S. Pöhlmann (Göttingen, Germany) and cultured in DMEM supplemented with 100 units mL^{-1} penicillin, 120 μg mL^{-1} streptomycin, 2×10^{-3} M L-glutamine, 1× nonessential amino acids (Sigma), and 10% FCS.

Lentiviral pseudotypes harbouring glycoproteins from Influenza, Lyssa, Rabies, Ebola, Marburg, SARS, and Lassa virus were obtained via cotransfection of HEK293T cells (450 000 cells, seeded in 6-well format) with (1) a crippled lentiviral vector encoding firefly luciferase and lacking a large part of env gene (pNLEnv-1)[60] and (2) expression plasmids for the respective viral glycoproteins, as described for Influenza H5N1 pseudotypes,[61] Lyssa and Rabies,[62] Ebola and Marburg,[65] SARS,[63] and Lassa.[64] Pseudotypes were harvested 2 d later, aliquoted and stored at –80 °C.

10 000 HEK293T cells (for Influenza pseudoparticles), 10 000 Huh7 cells (for Lyssa, Rabies, Ebola, and SARS pseudotyped lentiviruses), or 6000 Vero E6 cells (for Lassa pseudoparticles) were seeded the day before infection in 96-well plates. Next they were treated with 50 μg mL^{-1} of the polymers for 1 h at 37 °C before the cells were infected with the respective pseudoparticles. After 2–3 d, infection rates were determined by quantifying firefly luciferase activity in cellular lysates using the Luciferase Assay System (Promega, Madison, USA). All values represent % infection rates relative to control infections containing no polyanion (100%). Reporter gene activities (relative light units per second; RLU s^{-1}) were derived from triplicate infections minus background activities derived from uninfected cells.

Effect of Polymers on Human Adenovirus Infection: The E1-deleted replication-deficient human adenovirus type 5-based vector containing a human cytomegalovirus (HCMV) promoter-controlled enhanced green

© 2017 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

fluorescent protein (EGFP) expression cassette was produced on N52.E6 cells,[66] purified by one discontinuous and one continuous CsCl density gradient and subsequent size-exclusion chromatography (disposable PD-10, Amersham). The physical particle titer was determined by particle lysis and OD260 and confirmed by slot-blotting.[67] To assess effects of the polymers on virus infectivity, the vector was titrated with 20 mg L^{-1} of the polymers and incubated 10 min at 37 °C. 1 d prior to infection, 10^5 A549 cells (cultivated in MEM supplemented with 10% FCS, 100 units mL^{-1} penicillin, and 120 µg mL^{-1} streptomycin) per well were seeded in a 24-well format. Duplicate infection was performed with 500 multiplicity of infection (MOI) of the pretreated virus. EGFP expression was analyzed using a Beckman–Coulter Gallios flow cytometer 1 d post transduction.

Virus Inhibition Data Analyses: Viral inhibition data are expressed in % value relative to the level of infection in noninhibited samples (cell + virus only) and are reported as mean ± standard error derived from two independent experiments performed in triplicate for each datapoint.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

F.S., C.F.R., A.R., and J.A.M. contributed equally to this work. The authors wish to acknowledge financial support from the Danish Council for Independent Research, Technology and Production Sciences, Denmark (ANZ; project DFF: 4184-00177) and a grant from Deutsche Forschungsgemeinschaft (JM, project CRC 1279).

Conflict of Interest

The authors declare no conflict of interest.

Keywords

broad spectrum antivirals, microbicides, polyanions, polymers, Zika virus

Received: June 14, 2017
Revised: August 7, 2017
Published online: September 25, 2017

[1] T. Burnouf, J. Emmanuel, D. Mbanya, M. El-Ekiaby, W. Murphy, S. Field, J. P. Allain, *Lancet* **2014**, *384*, 1347.
[2] N. Afdhal, S. Zeuzem, P. Kwo, M. Chojkier, N. Gitlin, M. Puoti, M. Romero-Gomez, J. P. Zarski, K. Agarwal, P. Buggisch, G. R. Foster, N. Brau, M. Buti, I. M. Jacobson, G. M. Subramanian, X. Ding, H. M. Mo, J. C. Yang, P. S. Pang, W. T. Symonds, J. G. McHutchison, A. J. Muir, A. Mangia, P. Marcellin, ION-1 Investigators, *N. Engl. J. Med.* **2014**, *370*, 1889.
[3] E. De Clercq, G. D. Li, *Clin. Microbiol. Rev.* **2016**, *29*, 695.
[4] J. J. Feld, I. M. Jacobson, C. Hezode, T. Asselah, P. J. Ruane, N. Gruener, A. Abergel, A. Mangia, C. L. Lai, H. L. Y. Chan, F. Mazzotta, C. Moreno, E. Yoshida, S. D. Shafran, W. J. Towner, T. T. Tran, J. McNally, A. Osinusi, E. Svarovskaia, Y. Zhu, D. M. Brainard, J. G. McHutchison, K. Agarwal, S. Zeuzem, AON-1 Investigators, *N. Engl. J. Med.* **2015**, *373*, 2599.
[5] E. Bekerman, S. Einav, *Science* **2015**, *348*, 282.
[6] D. Baud, D. Musso, M. Vouga, M. P. Alves, N. Vulliemoz, *Am. J. Reprod. Immunol.* **2017**, *77*, e12614.
[7] D. H. Hamer, M. E. Wilson, J. Jean, L. H. Chen, *Curr. Infect. Dis. Rep.* **2017**, *19*, 16.
[8] VivaGel, http://www.starpharma.com/the_vivagel_condom (accessed: August 2017).
[9] J. S. Manning, A. J. Hackett, N. B. Darby Jr., *Appl. Microbiol.* **1971**, *22*, 1162.
[10] K. Cornetta, W. F. Anderson, *J. Virol. Methods* **1989**, *23*, 187.
[11] M. M. Kaplan, T. J. Wiktor, R. F. Maes, J. B. Campbell, H. Koprowski, *J. Virol.* **1967**, *1*, 145.
[12] M. Yolamanova, C. Meier, A. K. Shaytan, V. Vas, C. W. Bertoncini, F. Arnold, O. Zirafi, S. M. Usmani, J. A. Muller, D. Sauter, C. Goffinet, D. Palesch, P. Walther, N. R. Roan, H. Geiger, O. Lunov, T. Simmet, J. Bohne, H. Schrezenmeier, K. Schwarz, L. Standker, W. G. Forssmann, X. Salvatella, P. G. Khalatur, A. R. Khokhlov, T. P. Knowles, T. Weil, F. Kirchhoff, J. Munch, *Nat. Nanotechnol.* **2013**, *8*, 130.
[13] C. Meier, T. Weil, F. Kirchhoff, J. Munch, *WIRES Nanomed. Nanobi.* **2014**, *6*, 438.
[14] K. Ichiyama, C. Yang, L. Chandrasekaran, S. Liu, L. Rong, Y. Zhao, S. Gao, A. Lee, K. Ohba, Y. Suzuki, Y. Yoshinaka, K. Shimotohno, K. Miyakawa, A. Ryo, J. Hedrick, N. Yamamoto, Y. Y. Yang, *Macromolecules* **2016**, *49*, 2618.
[15] B. B. Hsu, S. Yinn Wong, P. T. Hammond, J. Chen, A. M. Klibanov, *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 61.
[16] P. Desomer, E. De Clercq, A. Billiau, E. Schonne, M. Claesen, *J. Virol.* **1968**, *2*, 878.
[17] P. Desomer, E. De Clercq, A. Billiau, E. Schonne, M. Claesen, *J. Virol.* **1968**, *2*, 886.
[18] A. A. A. Smith, M. B. L. Kryger, B. M. Wohl, P. Ruiz-Sanchis, K. Zuwala, M. Tolstrup, A. N. Zelikin, *Polym. Chem.* **2014**, *5*, 6407.
[19] M. Luscher-Mattli, *Antiviral Chem. Chemother.* **2000**, *11*, 249.
[20] D. Sepulveda-Crespo, R. Gomez, F. J. De La Mata, J. L. Jimenez, M. A. Munoz-Fernandez, *Nanomedicine* **2015**, *11*, 1481.
[21] B. Krust, C. Callebaut, A. G. Hovanessian, *AIDS Res. Hum. Retroviruses* **1993**, *9*, 1087.
[22] A. Vaillant, J. M. Juteau, H. Lu, S. Liu, C. Lackman-Smith, R. Ptak, S. Jiang, *Antimicrob. Agents Chemother.* **2006**, *50*, 1393.
[23] E. De Clercq, *Nat. Rev. Drug Discovery* **2002**, *1*, 13.
[24] E. De Clercq, *JAIDS, J. Acquired Immune Defic. Syndr.* **1991**, *4*, 207.
[25] G. Liu, S. Xiong, Y. F. Xiang, C. W. Guo, F. Ge, C. R. Yang, Y. J. Zhang, Y. F. Wang, K. Kitazato, *Arch. Virol.* **2011**, *156*, 1359.
[26] G. Moad, E. Rizzardo, S. H. Thang, *Aust. J. Chem.* **2012**, *65*, 985.
[27] S. Monge, B. Canniccioni, A. Graillot, J. J. Robin, *Biomacromolecules* **2011**, *12*, 1973.
[28] G. L. Patrick, *An Introduction to Medicinal Chemistry*, 4th edition, Oxford University Press Inc., New York **2013**.
[29] D. G. Anderson, D. M. Lynn, R. Langer, *Angew. Chem., Int. Ed.* **2003**, *42*, 3153.
[30] J. J. Green, R. Langer, D. G. Anderson, *Acc. Chem. Res.* **2008**, *41*, 749.
[31] S. Telwatte, K. Moore, A. Johnson, D. Tyssen, J. Sterjovski, M. Aldunate, P. R. Gorry, P. A. Ramsland, G. R. Lewis, J. R. Paull, S. Sonza, G. Tachedjian, *Antiviral Res.* **2011**, *90*, 195.
[32] T. Burnouf, M. Radosevich, *Blood Rev.* **2000**, *14*, 94.
[33] A. L. Zydney, *Biotechnol. Bioeng.* **2009**, *103*, 227.
[34] G. W. A. Dick, S. F. Kitchen, A. J. Haddow, *Trans. R. Soc. Trop. Med. Hyg.* **1952**, *46*, 509.
[35] J. A. Müller, M. Harms, A. Schubert, B. Mayer, S. Jansen, J.-P. Herbeuval, D. Michel, T. Mertens, O. Vapalahti, J. Schmidt-Chanasit, J. Münch, *Med. Microbiol. Immunol.* **2017**, *206*, 175.
[36] M. Aubry, V. Richard, J. Green, J. Broult, D. Musso, *Transfusion* **2016**, *56*, 33.

© 2017 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

[37] R. W. Driggers, C. Y. Ho, E. M. Korhonen, S. Kuivanen, A. J. Jaaskelainen, T. Smura, A. Rosenberg, D. A. Hill, R. L. DeBiasi, G. Vezina, J. Timofeev, F. J. Rodriguez, L. Levanov, J. Razak, P. Iyengar, A. Hennenfent, R. Kennedy, R. Lanciotti, A. du Plessis, O. Vapalahti, *N. Engl. J. Med.* **2016**, *374*, 2142.
[38] X. Wei, J. M. Decker, H. Liu, Z. Zhang, R. B. Arani, J. M. Kilby, M. S. Saag, X. Wu, G. M. Shaw, J. C. Kappes, *Antimicrob. Agents Chemother.* **2002**, *46*, 1896.
[39] A. N. Zelikin, E. S. Trukhanova, D. Putnam, V. A. Izumrudov, A. A. Litmanovich, *J. Am. Chem. Soc.* **2003**, *125*, 13693.
[40] M. Baba, E. De Clercq, D. Schols, R. Pauwels, R. Snoeck, C. Van Boeckel, G. Van Dedem, N. Kraaijeveld, P. Hobbelen, H. Ottenheijm, F. Den Hollander, *J. Infect. Dis.* **1990**, *161*, 208.
[41] M. Ito, M. Baba, K. Hirabayashi, T. Matsumoto, M. Suzuki, S. Suzuki, S. Shigeta, E. De Clercq, *Eur. J. Clin. Microbiol. Infect. Dis.* **1989**, *8*, 171.
[42] M. R. Proffitt, S. A. Schindler, *Clin. Diagn. Virol.* **1995**, *4*, 175.
[43] T. Lion, *Clin. Microbiol. Rev.* **2014**, *27*, 441.
[44] J. C. Hierholzer, *Clin. Microbiol. Rev.* **1992**, *5*, 262.
[45] C. W. Tan, I. C. Sam, W. L. Chong, V. S. Lee, Y. F. Chan, *Antiviral Res.* **2017**, *143*, 186.
[46] S. Ghezzi, L. Cooper, A. Rubio, I. Pagani, M. R. Capobianchi, G. Ippolito, J. Pelletier, M. C. Meneghetti, M. A. Lima, M. A. Skidmore, V. Broccoli, E. A. Yates, E. Vicenzi, *Antiviral Res.* **2017**, *140*, 13.
[47] L. J. Yockey, L. Varela, T. Rakib, W. Khoury-Hanold, S. L. Fink, B. Stutz, K. Szigeti-Buck, A. Van den Pol, B. D. Lindenbach, T. L. Horvath, A. Iwasaki, *Cell* **2016**, *166*, 1247.
[48] R. Uraki, K. A. Jurado, J. Hwang, K. Szigeti-Buck, T. Horvath, A. Iwasaki, E. Fikrig, *J. Infect. Dis.* **2017**, *215*, 1720.
[49] V. A. Kostyuchenko, E. X. Y. Lim, S. J. Zhang, G. Fibriansah, T. S. Ng, J. S. G. Ooi, J. Shi, S. M. Lok, *Nature* **2016**, *533*, 425.
[50] D. Sirohi, Z. Chen, L. Sun, T. Klose, T. C. Pierson, M. G. Rossmann, R. J. Kuhn, *Science* **2016**, *352*, 467.
[51] E. Chertova, J. W. Bess, B. J. Crise, R. C. Sowder, T. M. Schaden, J. M. Hilburn, J. A. Hoxie, R. E. Benveniste, J. D. Lifson, L. E. Henderson, L. O. Arthur, *J. Virol.* **2002**, *76*, 5315.
[52] P. Zhu, J. Liu, J. Bess, E. Chertova, J. D. Lifson, H. Grise, G. A. Ofek, K. A. Taylor, K. H. Roux, *Nature* **2006**, *441*, 847.
[53] M. Danial, H.-A. Klok, *Macromol. Biosci.* **2015**, *15*, 9.
[54] M. Al-Mahtab, M. Bazinet, A. Vaillant, *PloS one* **2016**, *11*, e0156667.
[55] D. I. Bernstein, N. Goyette, R. Cardin, E. R. Kern, G. Boivin, J. Ireland, J.-M. Juteau, A. Vaillant, *Antimicrob. Agents Chemother.* **2008**, *52*, 2727.
[56] F. Noordeen, A. Vaillant, A. R. Jilbert, *Antimicrob. Agents Chemother.* **2013**, *57*, 5291.
[57] C. Urbinati, P. Chiodelli, M. Rusnati, *Molecules* **2008**, *13*, 2758.
[58] A. Papkalla, J. Munch, C. Otto, F. Kirchhoff, *J. Virol.* **2002**, *76*, 8455.
[59] J. Munch, E. Rucker, L. Standker, K. Adermann, C. Goffinet, M. Schindler, S. Wildum, R. Chinnadurai, D. Rajan, A. Specht, G. Gimenez-Gallego, P. C. Sanchez, D. M. Fowler, A. Koulov, J. W. Kelly, W. Mothes, J. C. Grivel, L. Margolis, O. T. Keppler, W. G. Forssmann, F. Kirchhoff, *Cell* **2007**, *131*, 1059.
[60] F. Maldarelli, M. A. Martin, K. Strebel, *J. Virol.* **1991**, *65*, 5732.
[61] S. W. Liu, R. M. Li, R. T. Zhang, C. C. S. Chan, B. M. Xi, Z. B. Zhu, J. Yang, V. K. M. Poon, J. Zhou, M. Chen, J. Munch, F. Kirchhoff, S. Pleschka, T. Haarmann, U. Dietrich, C. G. Pan, L. Y. Du, S. B. Jiang, B. J. Zheng, *Eur. J. Pharmacol.* **2011**, *660*, 460.
[62] T. Federici, R. Kutner, X.-Y. Zhang, H. Kuroda, N. Tordo, N. M. Boulis, J. Reiser, *Genet. Vaccines Ther.* **2009**, *7*, 1.
[63] T. Gramberg, H. Hofmann, P. Moller, P. F. Lalor, A. Marzi, M. Geier, M. Krumbiegel, T. Winkler, F. Kirchhoff, D. H. Adams, S. Becker, J. Munch, S. Pohlmann, *Virology* **2005**, *340*, 224.
[64] L. R. Whitby, A. M. Lee, S. Kunz, M. B. A. Oldstone, D. L. Boger, *Bioorg. Med. Chem. Lett.* **2009**, *19*, 3771.
[65] X. Ji, G. G. Olinger, S. Aris, Y. Chen, H. Gewurz, G. T. Spear, *J. Gen. Virol* **2005**, *86*, 2535.
[66] G. Schiedner, S. Hertel, S. Kochanek, *Hum. Gene Ther.* **2000**, *11*, 2105.
[67] F. Kreppel, V. Biermann, S. Kochanek, G. Schiedner, *Hum. Gene Ther.* **2002**, *13*, 1151.

© 2017 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim