MAJOR ARTICLE

A New Class of Synthetic Peptide Inhibitors Blocks Attachment and Entry of Human Pathogenic Viruses

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Many enveloped viruses, including herpes viruses, hepatitis B virus (HBV), and hepatitis C virus (HCV), and human immunodeficiency virus (HIV), are among the most important human pathogens and are often responsible for coinfections involving ≥2 types of viruses. However, therapies that are effective against multiple virus classes are rare. Here we present a new class of synthetic anti-lipopolysaccharide peptides (SALPs) that bind to heparan sulfate moieties on the cell surface and inhibit infection with a variety of enveloped viruses. We demonstrate that SALPs inhibit entry of human immunodeficiency virus type 1 (HIV-1), herpes simplex virus (HSV) 1 and 2, HBV, and HCV to their respective host cells. Despite their high antiviral efficiency, SALPs were well tolerated, and neither toxicity nor measurable inhibitor-induced adverse effects were observed. Since these broad-spectrum antiviral peptides target a host cell rather than a viral component, they may also be useful for suppression of viruses that are resistant to antiviral drugs.

With onset of the worldwide AIDS epidemic in the 1980s, the pressing need for highly selective antiviral drugs to counter human immunodeficiency virus (HIV) infection not only resulted in novel combinatorial therapy approaches, such as highly active antiretroviral therapy [1], it also considerably progressed the development of drugs against other viruses [2, 3]. To date, these efforts have resulted in about 50 potent and already licensed antiviral drugs [3]. For the most part, these drugs target specific virus families and frequently select resistant viral variants.

The emergence of drug-resistant viruses, including multidrug-resistant strains, represents a significant problem in current clinical practice that needs to be addressed with high priority [4, 5]. In addition, very few antiviral strategies targeting several virus families are available so far. Moreover, advanced antivirals should be characterized by minimal adverse effects and, ideally, by high bioavailability [6]. To fulfill all these tasks, novel classes of antivirals are needed.

One option to avoid the selection of resistant virus variants is to target essential host cell components, a strategy that may also yield broad-spectrum antiviral agents acting against >1 virus family. Human pathogenic viruses frequently belong to the group of enveloped viruses that enter their host cell by fusing the viral envelope with cellular membranes, either at the plasma membrane or within the endosome. Therefore, compounds targeting components of either the host cell membrane or the host cell–derived viral envelope have been shown to have high potential to block entry of multiple classes of enveloped viruses [7, 8]. In addition, specific entry inhibitors are available, such as

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the T20 peptide for the treatment of HIV infection and the myristoylated peptide Myrcludex B [9], which is currently developed for the treatment of hepatitis B and D.

We found that synthetic anti-lipopolysaccharide (LPS) peptides (SALPs), which have recently been shown to protect mice from lethal septic shock [10], bind to heparan sulfate moieties on the plasma membrane. This new class of peptides is based on the LPS-binding domain of the Limulus anti-LPS factor [11], which neutralizes LPS and thus blocks its immunopathological consequences in vitro and in vivo [10].

In the present study, we investigated the antiviral potential of SALPs. We demonstrate that SALPs block entry of a variety of human pathogenic enveloped viruses, such as HIV-1, HBV, HCV, and HSV 1 and 2 by blocking heparan sulfate on the host cell plasma membrane, which serves as a the docking molecule for these pathogens. Therefore, SALPs represent an interesting new class of antiviral agents.

MATERIALS AND METHODS

Detailed materials and methods are contained in the supplementary materials, available at http://jid.oxfordjournals.org.

SALPs and Control Peptide

The following peptides were used in this study: Pep19-2.5 (GCKKYRRFRWKFKGKFWFWG), Pep19-4 (GKKYRRFRWKFKFGKWFWFG), and a control peptide (CPAQRKEETFKKYRA).

HIV-1 Infection

Infectious HIV-1 stocks of the CCR5-tropic strain BaL [12] and the CXCR4-tropic strain NL4-3 [13] were harvested 3 days after transfection of 4×10^6 293T cells. For HIV-1 infection, 2×10^7 PM-1 cells were incubated at 37°C for 3 hours with 5 ng p24 of HIV-1 in suspension, in the presence or absence of 20 μg/mL SALP or control peptide. After removal of the inoculum, 5×10^5 infected cells/mL were further cultured with peptides. For the luciferase infection assay, 1×10^4 TZM-bl cells were treated with a dilution series of SALP or control peptide prior to addition of 5 ng HIV-1 BaL or NL4-3 for 4 hours at 37°C. After 24 hours, luciferase expression was measured in cell lysates. To determine HIV-1 attachment, TZM-bl cells were seeded to confluency and pretreated with 20 μg/mL peptide for 1 hour before incubation with 20 ng p24 of HIV-1 BaL for 30 minutes at 4°C. After thorough washing, cells were lysed with phosphate-buffered saline and 0.5% NP–40, and intracellular p24 levels were determined by p24 enzyme-linked immunosorbant assay (ELISA). The cell-cell-fusion assay was performed in principle as described elsewhere [14].

HSV Infection

Gradient purified HSV-1 and HSV-2 inocula were prepared and titrated in principle as previously described [15, 16]. [^{3}H]-thymidine–labeled HSV-1 was grown by adding 5 mCi [methyl-^{3}H]-thymidine–labeled medium to producer BHK-21 cells. Vero cells grown to confluency were infected at 37°C at the indicated multiplicity of infection (MOI). For attachment assays, cells were kept at 4°C.

HBV Infection

The HBV inoculum was concentrated from the supernatant of HepG2.2.15 cells cultivated in Williams E Medium, 5% fetal calf serum, 1% dimethyl sulfoxide (DMSO), using centrifugal filter devices (Centricon Plus-70, Biomax 100 000; Millipore, Bedford, MA). Titers of HBV stocks were determined as enveloped DNA–containing viral particles by sedimentation into a CsCl density-gradient, as described elsewhere [17, 18]. To test heparin binding, concentrated culture supernatants of HepG2.2.15 cells were applied to 1 mL HiTrap Heparin HP Columns (GE Healthcare). HepaRG cells were cultured and differentiated for 2 weeks as previously described [9], preincubated with peptides as indicated, and infected with HBV in medium containing 1.8% DMSO and 5% polyethylene glycol 8000 for at least 16 hours at 37°C [19].

HCV Infection

Recombinant HCV expressing firefly luciferase (HCVcc [Luc-Jc1; genotype 2a/2a]) [20, 21] and lentivirus-based HCV pseudoparticles (HCVpp; genotype 1b) [22] were produced. Huh7.5.1 cells seeded in 96-well plates (10^4 cells/well) were cultured 24 hours prior to experiments, in the presence or absence of peptides, for 1 hour at 37°C and infected at 37°C for 4 hours with HCVcc or HCVpp. A total of 48 hours after HCVcc and 72 hours after HCVpp infection, cells were lysed, and luciferase activity was determined [20, 23].

RESULTS

Inhibition of HIV-1 Infection by Synthetic Anti-LPS Peptides

We first analyzed the potential of SALPs to interfere with the HIV-1 life cycle. PM1 cells were infected with the R5-tropic strain BaL [12], washed, resuspended, and cultured in the presence of increasing concentrations of SALPs (2.5–20 μg/mL) or in medium alone as a control for the calculation of relative p24 antigen levels. Every second day, the culture medium was changed, cells were split, and fresh SALPs were added. The viability of the cells (measured by the AlamarBlue assay) and p24 antigen levels in the cell-free supernatants were determined at days 8, 10, and 12 after infection. Both SALPs, Pep19-2.5 and Pep19-4, blocked HIV-1 replication in a dose-dependent fashion, reaching inhibition rates ranging from 90% to 100% at 20 μg/mL (Figure 1*A* and 1*B*). Cell-viability

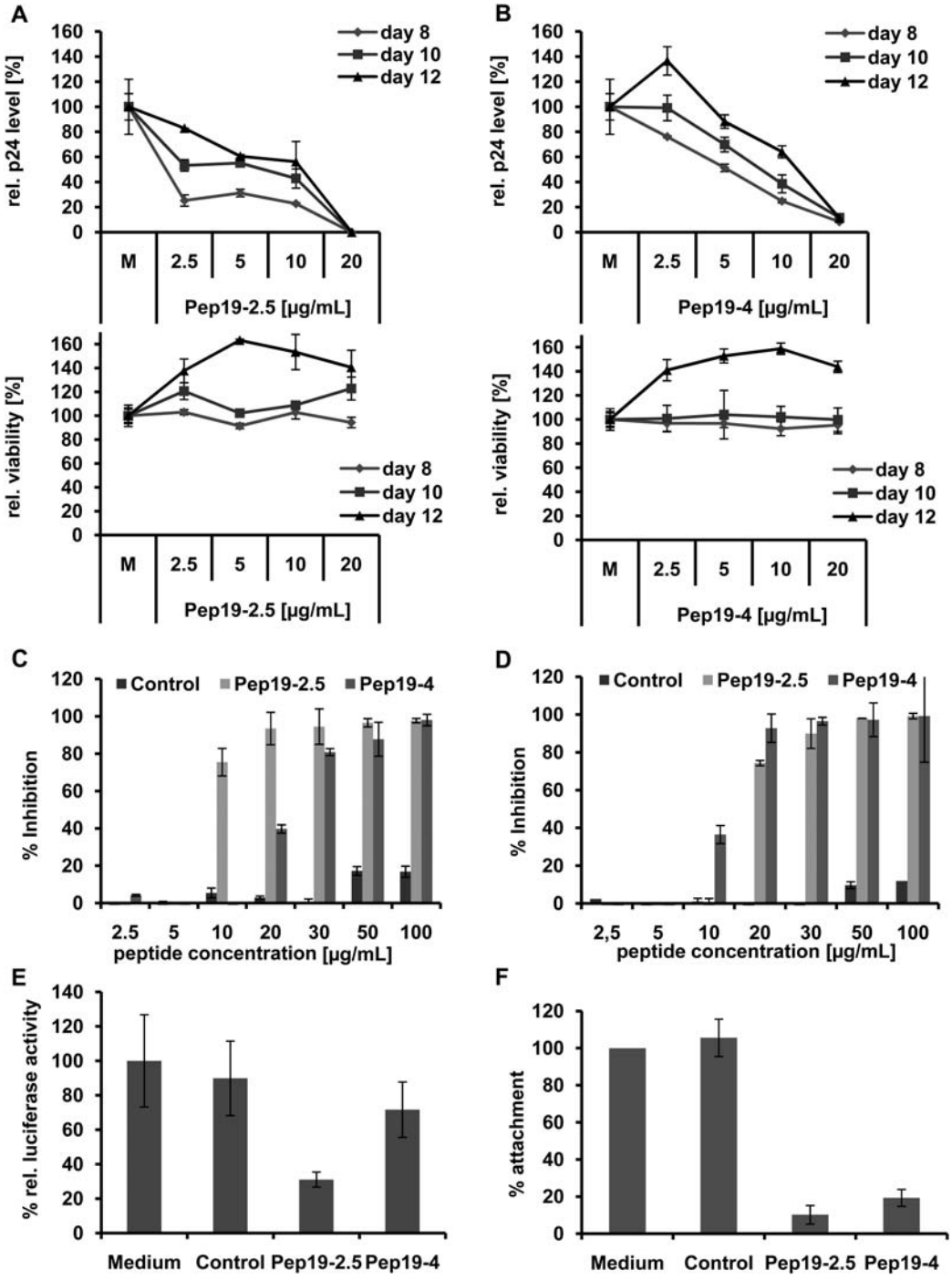

Figure 1. Inhibition of human immunodeficiency virus (HIV) infection at the level of attachment. PM1 cells were infected with R5-tropic HIV type 1 (HIV-1) BaL and cultivated in the presence Pep19-2.5 (*A*) or Pep19–4 (*B*) at the indicated concentrations or without medium only (M). p24 antigen levels in culture medium were determined at days 8, 10, and 12 (upper panel). Results of AlamarBlue viability assays for the same cultures are shown in the lower panels. Values for M-treated control cells were set to 100%. *C–F,* TZM-bl reporter cells, which express CD4, CCR5, and CXCR4 receptors on their cell surface and additionally contain a Tat-dependent HIV-1 long terminal repeat promoter-driven luciferase cassette, were treated with Pep19-2.5, Pep19-4, or control peptide and subsequently infected with R5-tropic HIV-1 BaL (*C*) or X4-tropic NL4-3 (*D*). Cells were lysed to determine luciferase activity 24 hours after infection. Inhibition of luciferase activity relative to untreated cells is given. *E*, 293T cells were cotransfected with pcTat and HIV-1 NL4-3 Env plasmids or with pcTat alone (negative control) to analyze effects on cell-cell fusion. After 20 hours, transfected 293T cells and Jurkat 1G5 cells were treated separately with 20 µg/mL of synthetic anti-lipopolysaccharide peptide or control peptide for 1 hour at 37°C, cocultivated for 24 hours, and analyzed for luciferase activity relative to untreated controls. *F,* TZM-bl cells were treated with 20 µg/mL of peptide and thereafter incubated with HIV-1 BaL at 4°C for 30 minutes. After washing, p24 antigen levels in whole-cell lysates were determined.

analyses did not reveal any adverse effect but showed improved cell viability because cytotoxic virus infection was prevented (Figure 1*A* and 1*B*).

The failure to detect SALP-induced cytotoxic effects was confirmed in more-elaborate toxicity studies, using Vero, TZM-bl, and Jurkat cells (Supplementary Figure 1A–E). These data suggested that a concentration of 20 μg/mL Pep19-2.5 or Pep19-4 can efficiently interfere with HIV-1 replication, whereas no cytotoxicity could be observed.

Since these peptides have been previously reported to bind to bacterial membrane components (ie, LPS) [10], we hypothesized that their antiviral effect may be due to the inhibition of an early step in the HIV-1 life cycle (eg, virus entry). We therefore used TZM-bl cells, which can be infected with both R5-tropic and X4-tropic viruses and allow the quantification of HIV infection via an integrated, Tat-responsive HIV-1 long terminal repeat (LTR)–luciferase reporter expression cassette [24]. After exposure to different concentrations of peptides for 1 hour, TZM-bl cells were infected for 4 hours with the R5-tropic strain BaL or the X4-tropic strain NL4-3 [13]. Cells were washed, further cultivated for another day in medium without SALPs, and immediately subjected to luciferase assay. As shown, de novo infection by R5 and X4 viruses was clearly impaired in cultures treated with Pep19-2.5 and Pep19-4 (Figure 1*C* and 1*D*) at half maximal inhibitory concentration (IC_{50}) in the low micromolar range (Table 1).

In another independent assay, we next explored the effect of Pep19-2.5 and Pep19-4 on HIV-1 Env-mediated cell-cell fusion. 293T donor cells were transiently transfected with vectors either expressing NL4-3 Env and the viral transactivator protein Tat or Tat alone (negative control). Fusion to Jurkat 1G5 target cells, which contain a stably integrated Tat-responsive HIV-1 LTR-luciferase construct [25], results in luciferase expression, serving as surrogate readout for membrane fusion [14]. At 20 hours after transfection, 293T donor cells and Jurkat 1G5 target cells were treated separately with SALP or control peptide for 1 hour before being mixed and cocultivated for 24 hours. Pep19-2.5 and, to a lesser extent, Pep19-4 inhibited the Env-mediated fusion of donor and target cells (Figure 1*E*). We thus concluded that the antiretroviral activity of SALPs interferes with an early event in the viral life cycle.

To address this in more detail, the capacity of SALPs to affect virus attachment to the host cell plasma membrane was directly investigated (Figure 1*F*). Therefore, TZM-bl cells pretreated with SALP or control peptide were exposed to HIV-1 BaL for 30 minutes. To remove unbound cell-free virus, cells were washed 5 times with cold PBS and subsequently lysed for the quantification of cell-associated virus by p24 antigen ELISA [26]. This procedure clearly demonstrated that Pep19-2.5 and Pep19-4 blocked the attachment of HIV-1 to SALP-treated host cells.

Table 1. Half Maximal Inhibitory Concentrations (IC_{50}) for Synthetic Anti-Lipopolysaccharide Peptide–Mediated Inhibition of Study Viruses

	Pep19-2.5		Pep19-4	
Virus	IC_{50} (μg/mL)	IC_{50} (μM)	IC_{50} (μg/mL)	IC_{50} (μM)
$HIV\text{-}1_{BaL}$	8.0	2.9	22	8.0
$HIV\text{-}1_{NL4\text{-}3}$	16	5.9	10	3.6
HSV-1	0.42	0.15	1.8	0.7
HCV_{pp}	40	15	37	14
HBV	1.0	0.37	ND	ND

Abbreviations: HBV, hepatitis B virus; HCV, hepatitis C virus; HIV-1, human immunodeficiency virus type 1; HSV-1, herpes simplex virus 1; ND, not determined.

SALPs Accumulate at the Surface of HIV Host Cells

Since Pep19-2.5 and Pep19-4 appeared to interfere with HIV-1 entry, we analyzed the cellular localization of a SALP. Following the exposure of TZM-bl cells to NBD-labeled Pep19-2.5, cells were washed and then fixed with paraformaldehyde, and plasma membranes and nuclei were stained. Subsequent confocal laser scanning microscopy revealed individual cells that appeared to be covered by Pep19-2.5 (Figure 2*A*). The recording of Z-stacks, followed by 3-dimensional reconstruction, demonstrated that Pep19-2.5 apparently accumulated at the cellular surface of TZM-bl cells (Figure 2*A*).

To visualize the localization of Pep19-2.5 at the plasma membrane at higher magnification, we next performed analyses at the ultrastructural level by using an ultrathin sectioning technique in combination with transmission electron microscopy. Dissolved Pep19-2.5 were encapsulated in capillary microtubes (200 μm diameter), fixed in situ, dehydrated, and embedded for subsequent staining of ultrathin sections and micrograph acquisition. As shown, Pep19-2.5 solution (20 μg/mL) formed small aggregates, which appeared to accumulate at the cell surface. No such phenomena were detected on sections showing the plasma membrane of untreated TZM-bl cells cultured on grid-marked dishes (Figure 2*B*). These results were confirmed when rhodamine-conjugated Pep19-2.5 was incubated with TZM-bl cells and subsequently detected by immunogold electron microscopy. Taken together, these data further supported the notion that antiviral SALPs accumulate at the cell surface and thus interfere with an early step in virus infection, presumably at the level of particle attachment.

SALPs Bind to Heparan Sulfate

At this point, we speculated that positively charged SALPs may bind to negatively charged heparan sulfate (HS), a widely expressed cell surface molecule [27] that is known to act as a

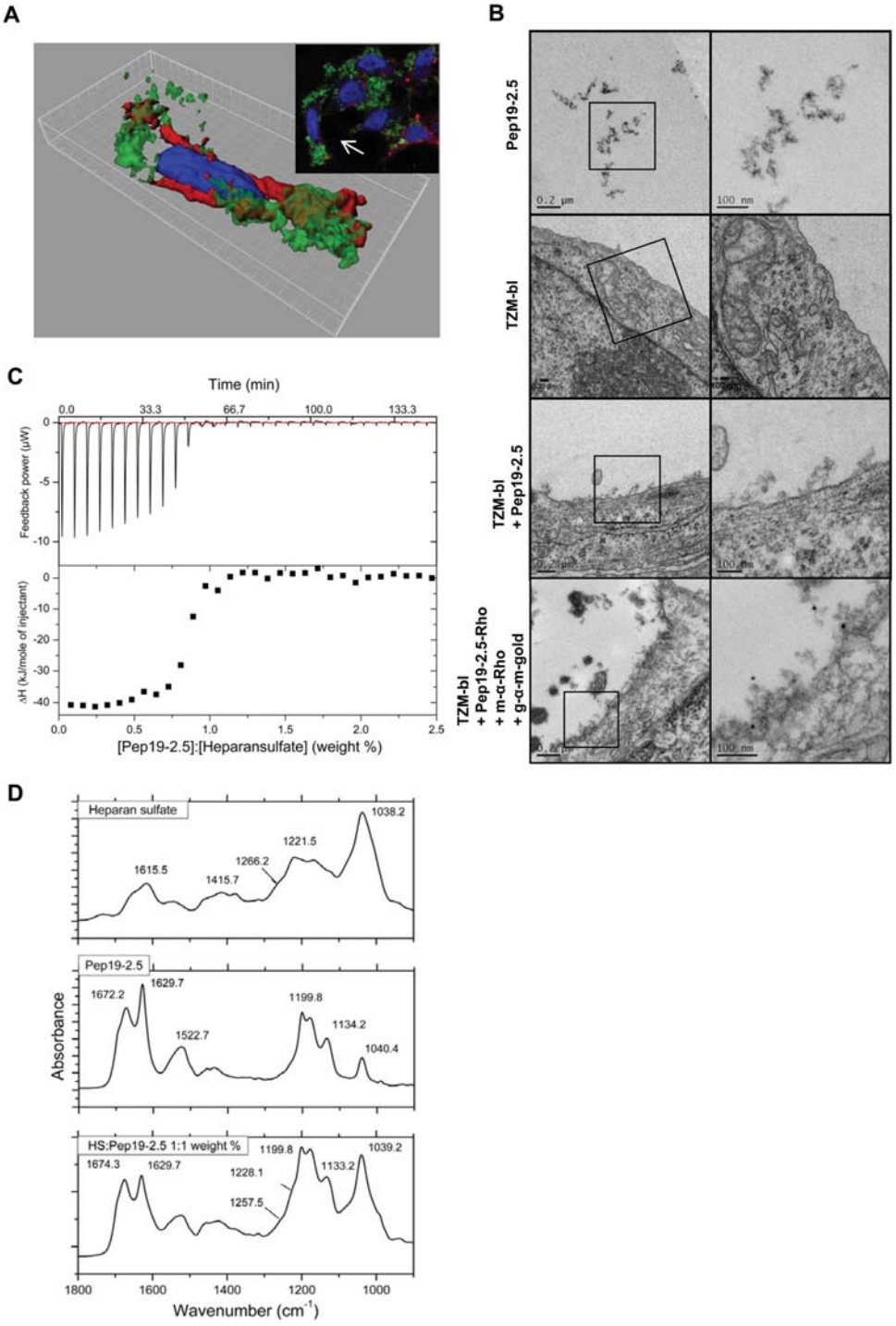

Figure 2. *A*, Subcellular localization of Pep19-2.5. 20 µg/mL of NBD-labeled Pep19-2.5 (green) was incubated with TZM-bl cells. Membranes were stained with DiI (red) and nuclei were stained with Draq5 (blue) for confocal microscopy. Three-dimensional reconstruction of Z-stacks of an exemplary cell is shown. *B*, Electron microscopy of a 20-µg/mL solution of Pep19-2.5 encapsulated into capillary microtubes, TZ-bl cells grown on grid-marked dishes, incubated with 20 µg/mL Pep19-2.5 or rhodamine-conjugated Pep19-2.5 detected by immunogold staining. *C*, Synthetic anti-lipopolysaccharide peptides binding to heparan sulfate was recorded by isothermal titration calorimetry. A total of 200 µg/mL of heparan sulfate was propounded in the microcalorimetric cell, and 2 mM Pep19-2.5 was titrated dropwise. Thermal differences were measured and plotted to the percentage weight of the components. *D*, Fourier-transform infrared spectra are shown for heparan sulfate (top panel), Pep19-2.5 (middle panel), and a 1:1 solution of both compounds (bottom panel). Automatic temperature scans of samples dissolved in 20 mM HEPES and placed in a CaF_2 cuvette were performed with an IFS-55 spectrometer (Bruker) between 10°C and 70°C. The "fingerprint" region in the spectral range of 1400 to 900 cm^{-1} containing information about the behavior of charged groups, such as sulfates and the sugar region, as well as about the Amid I vibrational band in the range of 1700 to 1600 cm^{-1}, which allows predication of the secondary structure, were monitored.

cellular HIV-1 docking site by binding the viral gp120 [28, 29] and also as docking molecule for a variety of other enveloped viruses. To verify this assumption, the interaction of Pep19-2.5 and HS was directly addressed by biophysical analysis. Isothermal titration calorimetry revealed a strong exothermic reaction when Pep19-2.5 was titrated to HS, corresponding to negative enthalpy changes (ΔH) of approximately −40 kJ/mol at the beginning of the experiment (Figure 2*C*). Moreover, the titration curve was a classic S-shaped binding curve, with saturation values at a HS:Pep19-2.5 ratio of 1:1 (weight percentage). This indicates strong Coulomb interaction between the 2 interaction partners, with neutralization of the HS charges due to their compensation by the 8 positively charged amino acids (R and K) in Pep19-2.5.

Comparison of Fourier-transform infrared spectra in the wavenumber range 1800 to 900 cm^{-1} for HS, Pep19-2.5, and a 1:1 solution of both compounds revealed an alteration of the peptide secondary structure and a characteristic change in the hydration status of the HS sulfate moieties due to a pronounced interaction of Pep19-2.5 and HS (Figure 2*D*).

SALPs Block HSV Attachment and Infection

Prior to receptor engagement, alphaherpesviruses exploit HS moieties on the cell surface and extracellular matrix for initial particle attachment [30–32]. In light of the binding data raised before, we investigated the effect of SALPs on de novo infection with HSV-1 and HSV-2. As shown by plaque-reduction assay (Figure 3*A*), the exposure of Vero cells to 20 μg/mL SALPs for 1 hour significantly blocked infection and cell-to-cell-spread by both HSV-1 and HSV-2. This inhibitory effect was not observed when the control peptide was used. These results were further confirmed by Western blots of extracts isolated from HSV-infected cultures, using either antibodies raised against the HSV-1 immediate early protein ICP0 or the HSV-2 major capsid component viral particle (Figure 3*B*).

To directly monitor SALP-dependent HSV particle attachment and virus entry, Vero cells were incubated in presence of SALP or control peptide for 1 hour as before, exposed for 2 hours on ice to [^{3}H] radiolabeled HSV-1, and further cultured for an additional hour at 37°C. To remove cell surface–bound virus particles, cells were subsequently treated with protease and thoroughly washed with PBS. Intracellular virions were quantified by direct scintillation counting of the respective Vero cell cultures. As shown, both Pep19-2.5 and Pep19-4 blocked the cellular uptake of HSV–1, reaching inhibition rates of nearly 100% in these experiments (Figure 3*C*). Importantly, when comparable cell cultures were harvested and counted directly after virus exposure and PBS washing, virus attachment was clearly diminished in a SALP-dependent manner (Figure 3*D*). The dose-dependence of this effect is shown in Figure 3*E*, and IC_{50} values in the submicromolar range were deduced (Table 1). These data demonstrated that HSV infection can be efficiently blocked by SALPs because of their interference with the initial binding of virions to HS moieties on host cell surfaces.

SALPs Inhibit HCV Entry and Infection

Highly sulfated HS represents a primary docking site for HCV [33, 34] before the virus interacts with its cell surface receptor molecules, namely CD81, scavenger receptor B1, claudin 1, and occludin, to enter hepatoma cells [20]. HCVpp and recombinant, infectious HCVcc represent state-of-the-art in vitro model systems to study HCV entry and infection, respectively [34]. To investigate whether the antiviral peptides were able to inhibit HCV entry, we first assessed their ability to interfere with HCVpp entry. Pep19-4 and Pep19-2.5 inhibited HCV entry in a dose-dependent manner (Figure 4*A* and 4*B*) at a calculated IC_{50} of around 40 μg/mL for both peptides (Table 1) without affecting cell viability (Supplementary Figure 1F). By using HCVcc for infection, we confirmed that both peptides inhibit HCVcc infection in a dose-dependent manner (Figure 4*C* and 4*D*), whereas control peptide affected neither HCVpp nor HCVcc entry. From these data, we concluded that SALPs interfere with initiation of the HCV entry process, which is dependent on binding to HS [33, 34].

SALPs Efficiently Inhibit HBV Entry by Blocking HS Interaction

Experimental evidence has been presented that HBV initiates infection of hepatocytes by binding to HS proteoglycans [35]. We therefore analyzed the antiviral activity of SALPs against HBV. Differentiated HepaRG cells that are susceptible to HBV infection in vitro [9] were treated before or after inoculation with HBV, using different concentrations of Pep19-2.5.

After entry into its host cell, HBV deposits a covalently closed circular DNA (cccDNA) molecule in the nucleus. cccDNA serves as a transcription template for HBV RNAs translated into HBV surface and core and its secreted form HBV e antigen (HBeAg), as well for pregenomic RNA, which is subsequently reverse transcribed into newly formed viral relaxed circular DNA genomes. All these markers of HBV infection [36, 37] were analyzed after infection with and infection without SALPs. Results showed that Pep19-2.5 very efficiently and dose dependently decreased all HBV infection markers in cells treated prior to HBV infection, whereas no effect was observed if cells were treated following HBV infection (Figure 5*A*–*D*). We thus concluded that Pep19-2.5 inhibits HBV entry into hepatocytes and has no influence on further steps of the HBV life cycle. This result was confirmed by SALP treatment of HuH7 cells containing replicating HBV after transfection of HBV plasmids (data not shown). The IC_{50} was determined to be around 1 μg/mL of Pep19–2.5. Up to 20 μg/mL, no cytotoxicity was observed (Supplementary Figure 1G). Results of tests involving a series of peptide variants indicated that the overall amount of positive charges and

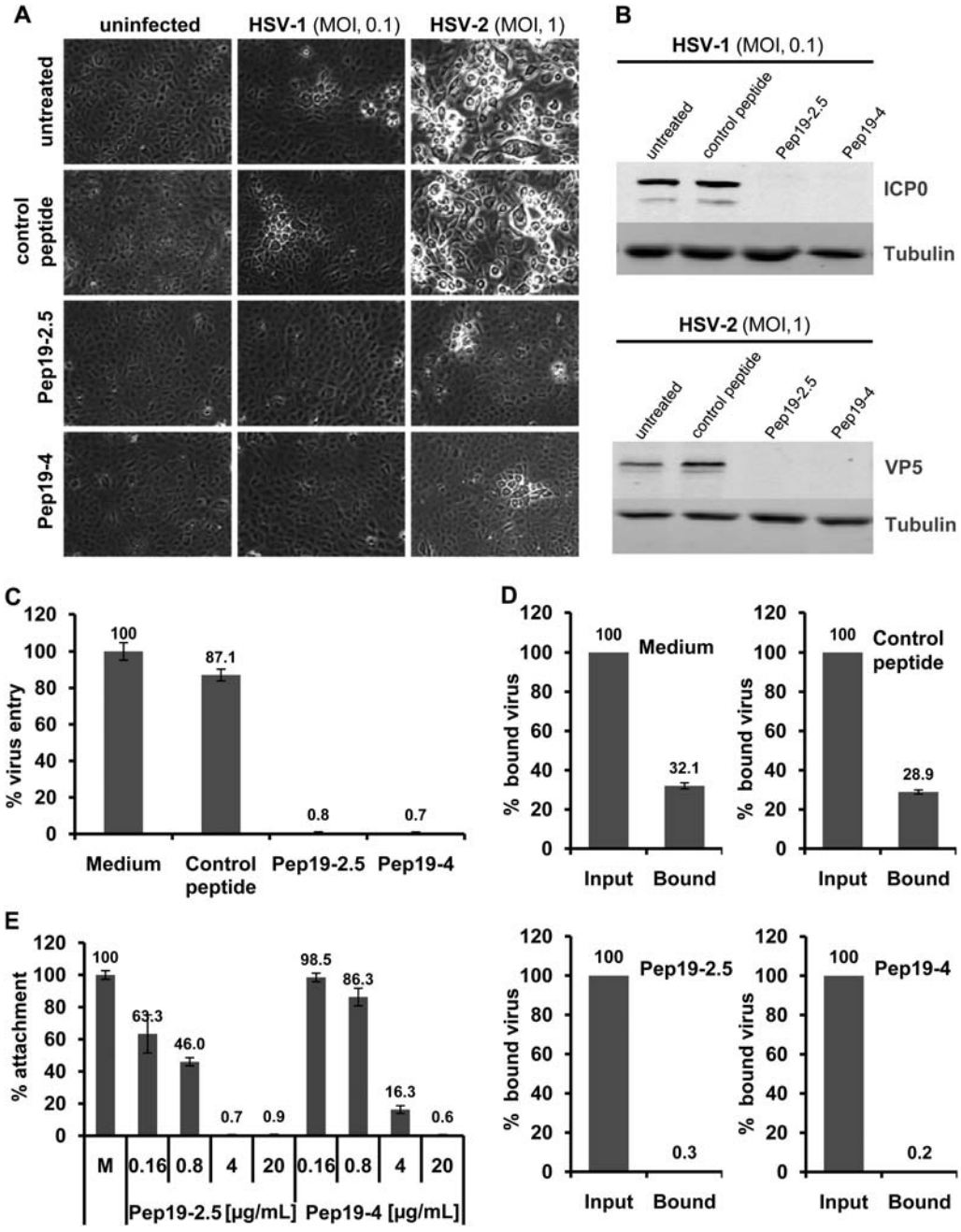

Figure 3. Inhibition of herpes simplex virus 1 (HSV-1) and 2 (HSV-2) replication. *A*, Vero cells were treated with 20 µg/mL of synthetic anti-lipopolysaccharide peptide (SALP) or control peptide as indicated and then incubated for 2 hours on ice with HSV-1 (multiplicity of infection [MOI], 0.1) or HSV-2 (MOI, 1) and for 18 hours at 37°C after washing. Phase-contrast images were recorded with a Zeiss Axiovert 200M microscope. *B*, Cell lysates were separated through sodium dodecyl sulfate polyacrylamide gel electrophoresis and analyzed by Western blotting for HSV-1 early protein ICP0 or the capsid protein viral particle of HSV-2 and tubulin as a loading control. *C*, To investigate attachment, [^{3}H]-radiolabeled HSV-1 virions were added to Vero cells without and with indicated peptides. Cells were treated with protease to digest bound but not entered virus particles and were harvested to count radioactivity with a Beckmann Coulter LS6500 scintillation counter. *D*, Blocking of attachment upon SALP treatment was monitored by washing and harvesting the cells directly after 2 hours of incubation with virus on ice. *E*, Dose dependence of the SALP-mediated inhibition of HSV-1 attachment. Abbreviation: M, medium.

their equal distribution over the peptide, rather than the peptides sequence or amphiphilicity, determine the inhibitory capacity of SALPs (Supplementary Figure 2).

Since we found the majority of rhodamine-labeled Pep19-2.5 bound to the surface of HepaRG cells (Figure 5*F*), we hypothesized that Pep19-2.5 prevented the interactions of

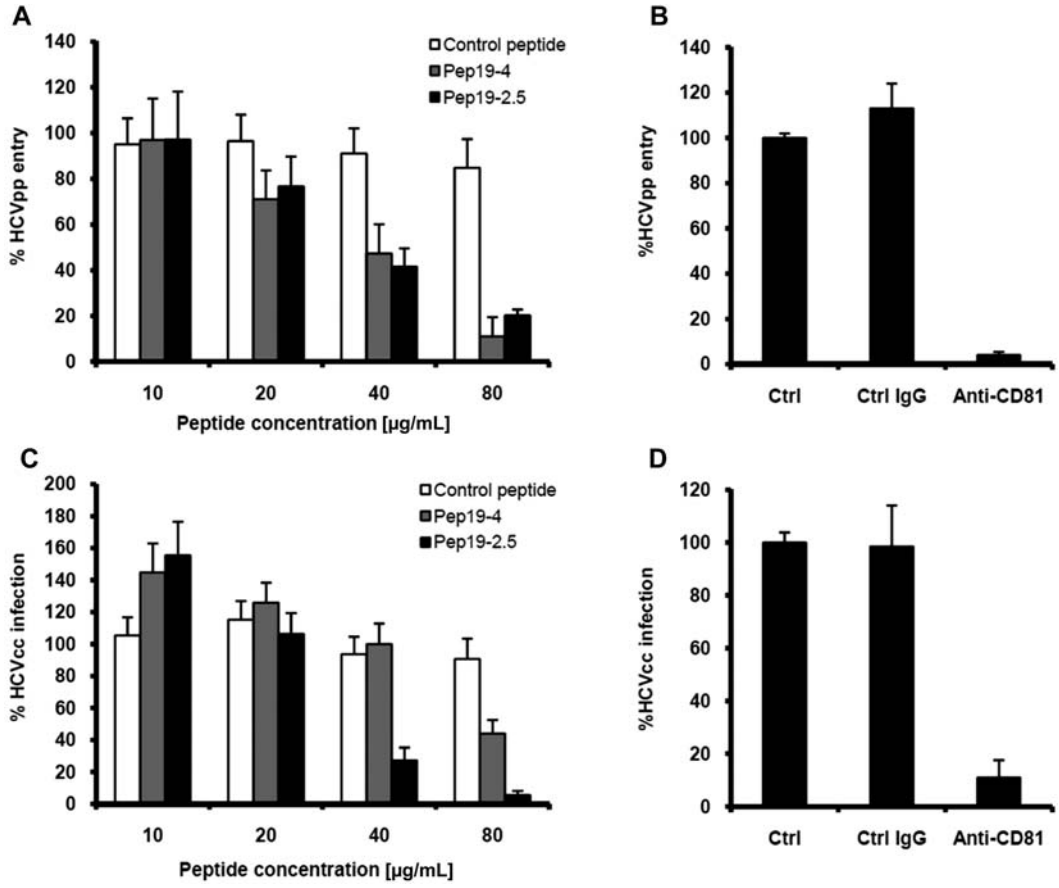

Figure 4. Synthetic anti-lipopolysaccharide peptides inhibit hepatitis C virus (HCV) entry into hepatoma cells. *A, B,* Huh7.5.1 cells were incubated with peptides 19-4 and 19-2.5 at indicated concentrations before infection with HCV pseudoparticles (HCVpp). After 72 hours, HCV infection was analyzed by quantification of luciferase activity in cell lysates. *C, D,* Huh7.5.1 cells were preincubated with peptides at the indicated concentrations before infection with HCV particles that are infectious in cell culture (HCVcc). After 48 hours, infection efficacy was analyzed by quantification of luciferase activity in cell lysates. Values for HCVpp or HCVcc infection in the absence of peptide were set to 100%. Anti-CD81 antibody and control mouse immunoglobulin G (IgG) antibody served as positive and negative controls (Ctrls), respectively.

HBV particles with HS proteoglycans on the hepatocyte surface. To test this, HBV virions incubated with or without Pep19-2.5 were applied to a heparin affinity chromatography column to determine their binding. As expected [35], most HBV virions bound to heparin-sepharose under physiological salt conditions and could be eluted with a high concentration of NaCl (Figure 5*E*). HBV particle binding to heparin was reduced by 60% when particles were loaded together with Pep19-2.5 (Figure 5*E*). To determine whether Pep19-2.5 interacted with heparin or with the HBV particles, we loaded the heparin-sepharose column with Pep19-2.5 and washed off unbound peptides before HBV particles were loaded (Figure 5*E*). Since we observed an equal reduction of HBV binding, we concluded that Pep19-2.5 competes with HBV for binding to HS. Therefore, our results show that SALPs inhibit initial binding of HBV to the cell surface by direct interaction to HS.

DISCUSSION

Over the previous 2 decades, the development of novel therapies for the treatment of human pathogenic viruses provided an entire array of new antiviral drugs [2]. So far, inhibitor development focused mainly on small-molecule drugs. However, this is gradually changing, as biologicals, including nucleic acids (eg, microRNA), antibodies, and peptides, are being tested in viral disease [38].

The targeting of viral factors will result in inhibitors with high specificity and potency, low toxicity, and a low resistance barrier. In comparison, the latter approach may yield broad-spectrum antivirals with minimal or no risk of resistance development but with a risk of lower antiviral potency and comparably higher toxicity. Obviously, the latter approach may be particularly successful by targeting early interactions between virus and cell and, thereby, virus entry [39].

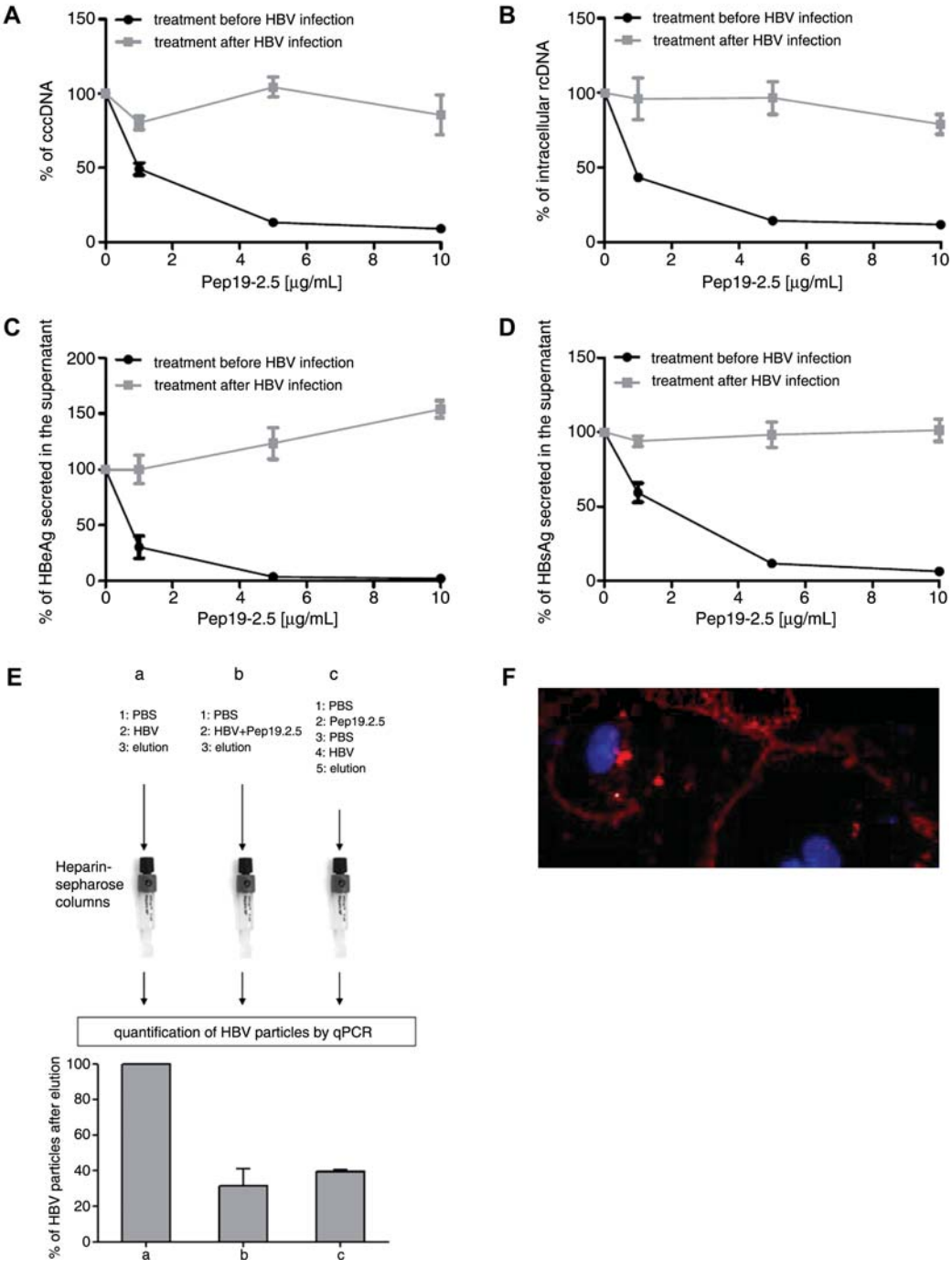

Figure 5. Pep19-2.5 inhibits hepatitis B virus (HBV) entry into hepatocytes. *A–D*, Differentiated HepaRG cells were infected with wild-type HBV particles at a multiplicity of infection of 200 viral particles per cell. Cells were treated with different concentrations of Pep19-2.5 either 24 hours before and during HBV inoculation or 4 days after infection. At day 11 after infection, cells were lysed, total intracellular DNA was extracted, and covalently closed circular DNA (cccDNA; *A*) and relaxed circular DNA (rcDNA; *B*) were analyzed by quantitative polymerase chain reaction (PCR). Cell culture media were analyzed by enzyme-linked immunosorbant assay for extracellular HBV core antigen (HBeAg; *C*) and HBV surface antigen (HBsAg; *D*) secretion. Values were set to 100% for the untreated cells. *E*, Pep19-2.5 blocks binding of HBV particles to heparin. *E-a*, Concentrated supernatants from HepG2.2.15 cells containing HBV particles were applied to heparin-sepharose affinity columns. After washing, enveloped HBV particles were eluted. The amount of HBV virions eluted was determined by quantitative real-time PCR for HBV DNA. *E-b,* The identical amount of HBV-containing inoculum was mixed with 50 µg/mL Pep19-2.5 before loading onto the column. *E-c*, Pep19-2.5 was bound to the column before the HBV inoculum was loaded. Values were set to 100% for condition a. *F*, Localization of Pep19-2.5 on HepaRG cells. HepaRG cells were incubated with rhodamine-labeled Pep19-2.5, fixed, and visualized by confocal microscopy. Nuclei were counterstained with DAPI. Abbreviation: PBS, phosphate-buffered saline.

In the present study, we report that a new class of synthetic anti-LPS peptides, termed SALPs, are characterized by broad-spectrum antiviral activity. We demonstrated that SALPs, particularly Pep19-2.5, significantly interfered with the de novo infection of various prototypic human pathogenic viruses, namely, HIV, HSV, HCV, and HBV, that are major health concerns.

SALPs showed low toxicity in all cell lines used. In an in vivo experiment, no SALP toxicity was reported when 150 μg of SALPs were administered intraperitoneally to 6-week-old C57BL/6 mice [10], while therapeutic effects are expected at 50 μg per mouse or 140 mg per human. This makes us confident that a therapeutic window exists for SALP-mediated antiviral therapies.

By using various biological and biophysical assays, we showed that SALPs bind to HS proteoglycan moieties covering the surface of cells [27]. SALPs represent multivalent inhibitors of varying viruses that, as a common denominator, use cell surface HS as a target for their initial attachment to the host cell [29, 35, 40]. Evidently, blocking virus attachment to the host cell would be a preferred antiviral strategy (eg, to prevent HBV or HCV reinfection after liver transplantation) because it is the first opportunity to curtail the viral life cycle.

The inhibitory effect of different SALPs (ie, Pep19-2.5 and Pep19-4), as well as a series of variant peptides, on virus entry was similar, suggesting that the overall positive charges and the equal distribution over the peptide, rather than peptide sequence and amphiphilicity, influence their antiviral activity (Supplementary Figure 2). Corresponding to the lower coverage of HuH7.5 cells with HS, concentrations to inhibit HCV entry and infection were higher than those needed for HBV, HSV, and HIV. Indeed, we have previously shown that HCV binding and entry to liver target cells requires a specific structure and conformation of highly sulfated heparan sulfate, including N-sulfo groups, and a minimum of 10–14 saccharide subunits [33, 41].

On the basis of their mode of action—the direct binding to HS—we predict that SALPs may also block the entry of further enveloped viruses. The wide distribution of HS may require high systemic doses, and SALPs may even bind to other negatively charged molecules on cell surfaces. However, SALPs may be advantageous in salvage therapies that aim at the treatment of multidrug-resistant viruses [4, 5]. Moreover, the topical application of SALPs as components of antiviral microbicides may contribute to the prevention of the sexual transmission of enveloped viruses [42].

Taken together, we have described antiviral synthetic peptides that prevent the attachment and entry of different human pathogenic enveloped viruses. SALPs target HS moieties on host cell surfaces and represent a new class of synthetic broad-spectrum antivirals that block multiple classes of viruses. While SALPs lack overt cytotoxicity in cell culture, further in vivo experiments and preclinical testing in animals are needed to prove their therapeutic value.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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