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A new class of synthetic anti-lipopolysaccharide peptides inhibits influenza A virus replication by blocking cellular attachment

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ABSTRACT

Influenza A viruses are a continuous threat to human health as illustrated by the 2009 H1N1 pandemic. Since circulating influenza virus strains become increasingly resistant against currently available drugs, the development of novel antivirals is urgently needed. Here, we have evaluated a recently described new class of broad-spectrum antiviral peptides (synthetic anti-lipopolysaccharide peptides; SALPs) for their potential to inhibit influenza virus replication *in vitro* and *in vivo*. We found that particularly SALP PEP 19-2.5 shows high binding affinities for the influenza virus receptor molecule, N-Acetylneuraminic acid, leading to impaired viral attachment and cellular entry. As a result, replication of several influenza virus subtypes (H7N7, H3N2 and 2009 pandemic H1N1) was strongly reduced. Furthermore, mice co-treated with PEP 19-2.5 were protected against an otherwise 100% lethal H7N7 influenza virus infection. These findings show that SALPs exhibit antiviral activity against influenza viruses by blocking virus attachment and entry into host cells. Thus, SALPs present a new class of broad-spectrum antiviral peptides for further development for influenza virus therapy.

1. Introduction

Influenza A viruses are a persistent threat to humans as illustrated by annual epidemics and sporadic pandemics. During annual epidemics, approximately three to five million cases of severe illness and 250,000–500,000 deaths are reported by the World Health Organization (WHO, 2009). Furthermore, occasional interspecies transmissions of avian viruses of the H5N1, H7N7 or recently the H7N9 subtypes may occur, resulting in high mortality rates in humans (Gao et al., 2013; Klenk et al., 2011; WHO, 2012).

The primary strategy to prevent influenza virus infection is vaccination. In case of an epidemic or pandemic, the production of new effective vaccines requires a certain time period and is therefore not immediately available. Consequently, antiviral drugs are essential to control illness and transmission, especially in the beginning of a novel influenza virus outbreak until an adequate vaccine becomes available. The adamantane derivatives (amantadine and rimantadine) and the neuraminidase inhibitors (zanamivir and oseltamivir) are both well-known and approved drugs against influenza viruses (De Clercq, 2006). The adamantane derivatives inhibit the influx of H^+ ions into the virion during the viral uncoating process by blocking the matrix protein 2 (M2) (Davies et al., 1964; Schnell and Chou, 2008). The treatment with zanamivir or oseltamivir prevents the release of newly assembled viral particles by competitive inhibition. Thereby, the cleavage of glycosidic linkages of N-Acetylneuraminic acids by the neuraminidase is reduced (Kim et al., 1997; Moscona, 2005; von Itzstein et al., 1993).

However, during the last decade, the emergence of drug resistant strains has limited the clinical use of these drugs. During the 2005/2006 influenza season, adamantine resistance rates rose up to 92.3% for H3N2 and 15.5% for seasonal H1N1 influenza viruses (Bright et al., 2006; Deyde et al., 2007) due to single mutations in the M2 ion channel protein (Hayden and de Jong, 2011; Schnell and Chou, 2008). Oseltamivir resistant H1N1 viruses started to spread during the 2007/2008 influenza season and reached almost 100% drug resistance in 2008/2009 (CDC, 2009; Dharan et al., 2009; Moscona, 2009) due to several mutations in the neuraminidase (Baranovich et al., 2011; Moscona, 2009). To date, almost 100% of the currently circulating 2009 pandemic H1N1 strains are resistant against adamantane derivatives, whereas no or little resistance was observed against zanamivir and oseltamivir, respectively (MMWR, 2012). Based on these viral surveillance and resistance

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data, the CDC currently does not recommend the use of adamantane derivatives. Instead, the updated guidelines recommend the immediate treatment of patients with either confirmed or suspected influenza with oseltamivir or zanamivir (Fiore et al., 2011).

Therefore, the development of novel antiviral strategies is urgently needed in order to be prepared against increasing antiviral resistances of circulating influenza virus strains.

In this study, we evaluated the potential of a new class of synthetic anti-lipopolysaccharide peptides (SALPs) to inhibit influenza virus replication. The SALPs are originally based on the LPS-binding domain of *Limulus* anti-lipopolysaccharide-factor (LALF) and have been recently re-constructed to increase their lipopolysaccharide (LPS) binding efficiency. These optimized SALPs interact with the LPS of Gram-negative bacteria to high affinity and protect against lethal septic shock in a mouse-sepsis-model. However, their antimicrobial and anti-inflammatory activity was not limited against Gram-negative but was also observed against Gram-positive bacteria (Gutsmann et al., 2010; Heinbockel et al., 2013; Kaconis et al., 2011; Schuerholz et al., 2012).

Very recently, it was described that SALPs additionally possess antiviral activities against some enveloped viruses, such as the human immunodeficiency virus (HIV), hepatitis B and C viruses (HBV, HCV) and herpes simplex viruses (HSV) by directly interacting with heparan sulfate, which is used by these viruses as a co-receptor molecule, and thereby inhibiting virus entry into host cells. Due to their potential to inhibit a variety of virus strains, they have been postulated to pose a promising new class of broad-spectrum antiviral peptides to combat various viral infections or co-infections (Krepstakies et al., 2012).

Novel antiviral approaches targeting very early stages of the viral life cycle, such as receptor recognition and cell entry as described with SALPs for other viruses would be highly desirable against influenza viruses.

In the present study, we therefore evaluated whether SALPs also possess antiviral activities against influenza A viruses *in vitro* and *in vivo*.

2. Materials and methods

2.1. Cells and viruses

Madin-Darby canine kidney (MDCK) and human lung carcinoma (A549) cells were grown in MEM (minimal essential medium; PAA) or DMEM (Dulbecco's modified Eagle Medium; PAA), respectively, supplemented with 10% FCS (fetal calf serum; PAA), 1% penicillin/streptomycin (PAA) and 1% L-Glutamine (PAA) at 5% CO_2 and 37 °C. Recombinant mouse-adapted SC35M (H7N7) virus, human A/Victoria/3/75 (H3N2) and A/Hamburg/NY1580/09 (2009 pandemic H1N1; pH1N1) strains were propagated in 11 day old embryonated chicken eggs or MDCK cells, respectively as described previously (Gabriel et al., 2011).

2.2. Antibodies

Primary antibodies used for Western blot analysis, immunofluorescence and immunohistochemistry assays include rabbit anti-H7N1 serum (Otte et al., 2011), mouse anti-PB2 (kindly provided by J. Ortin, CSIC, Madrid, Spain), rabbit anti-GAPDH (Cell signaling, 2118), mouse anti-pH1N1 serum and mouse anti-NP antibody (abcam, ab43821). Anti-mouse-HRP (Sigma, A4416) and anti-rabbit-HRP (Sigma, A8275) were used as secondary antibodies for Western blot analysis. For immunofluorescence and immunohistochemistry, anti-mouse-Alexa Fluor® 488 (Life Technologies, A21202), anti-mouse-Alexa Fluor® 647 (Life Technologies, A21235) or biotin-conjugated anti-rabbit (Jackson ImmunoResearch, 711-066-152) secondary antibodies were used.

2.3. Peptide synthesis

SALPs were synthesized by the solid-phase synthesis technique in an automatic peptide synthesizer (model 433 A; Applied Biosystems) as described before (Gutsmann et al., 2010). For these studies, SALPs were used with previously reported broad-spectrum antiviral activities as well as the non-positive charged control peptide PEP 19-CP (Krepstakies et al., 2012). Sequence and molecular weight of SALPs is presented in the Table 1. To study SALP function by immunofluorescence and electron microscopy as well as distribution in the murine airway, PEP 19-2.5 was conjugated with rhodamine at the N-terminus. Peptides were diluted in water and stocks of 1 or 5 mg/ml were stored at −80 °C. The purity of the peptides was higher than 95% as determined by HPLC. Peptide sequences were published by the European Patent Office (EP2108372) in October 2009.

2.4. Cytotoxicity assay

Potential cytotoxicity of SALPs was measured by an MTT Cell Proliferation Assay according to the manufacturer's instructions. Briefly, SALPs (20 or 50 μg/ml diluted in PBS) were added into the cell culture medium and applied to 80–90% confluent MDCK cells. After 2 days, cell viability was detected by using the MTT Cell Proliferation Assay-kit (ATCC) at similar conditions used for the plaque assays described below. Absorbance was measured at a wavelength of 590 nm and ranged from 0.7 to 1.4. Average values for the blank were subtracted and the absorbance of untreated control groups was set 100%. All experiments were performed at least four independent times.

2.5. Isothermal titration calorimetry

SALPs (5 or 10 mM in 20 mM Hepes) were titrated in 3 μl volumes every 5 min with N-Acetylneuraminic acids (0.05 mM in 20 mM Hepes, Sigma). The enthalpy change was determined by the MSC isothermal titration calorimeter (Microcal Inc.). The values determined by peptide titration alone were subtracted from the enthalpy change observed by titrating peptides with N-Acetylneuraminic acids. Experiments were performed at least twice.

2.6. Hemagglutination assay

To assess the effect of SALPs to inhibit virus hemagglutination to erythrocytes, we have used a hemagglutination assay. Therefore, mouse-adapted H7N7, human H3N2 or pH1N1 influenza virus was diluted in PBS (corresponding to 10^5–10^6 p.f.u.) containing 5 or 10 μg/ml of the respective SALP in a total volume of 50 μl. As controls, infected cells were either treated with PBS (control 1) or PEP 19-CP (control 2). Subsequently, the inoculum containing virus and SALP was added to the same volume (50 μl) of a 1% chicken erythrocyte suspension. After 45 min of incubation on ice, the grade of hemagglutination and/or hemagglutination inhibition was de-

Table 1
Peptide sequences of SALPs (PEP 19-2.5, PEP 19-4, PEP 19-8D and PEP 19-CP). Molecular weight is stated as Da.

Peptide	Sequence	MW
PEP 19-2.5	GCKKYRRFRWKFKGKFWFWG	2711
PEP 19-4	GKKYRRFRWKFKGKWFWFG	2750
PEP 19-8D	GFWFKGKWRFKKYRGGRYKKFRWKGKFWFG	3964
PEP 19-CP	SSNKSTTGSGETTTA	1427

tected. As a control, either virus or SALPs were incubated with erythrocytes only. Complete hemagglutination comparable to erythrocytes treated with virus in the absence of SALPs was set 100%. In contrast, absence of hemagglutination comparable to erythrocytes in the absence of virus was set 0%. The grade of hemagglutination inhibition upon SALP treatment was then directly compared to hemagglutinating wells without SALP treatment. All experiments were performed at least two independent times.

2.7. Immunofluorescence assays

To assess viral attachment on cell surface, A549 cells were seeded to approximately 50% confluency onto cover slips and incubated with 10^5 p.f.u. of the pH1N1 influenza virus strain for 30 min at 4 °C. Treatment with rhodamine-conjugated PEP 19-2.5 (20 μg/ml diluted in PBS) was analyzed in three different approaches: (i) pre-treatment: SALP was applied to cells 20 min prior virus inoculation, (ii) co-treatment: cells were incubated with virus and simultaneously co-treated with SALP and (iii) post-treatment: cells were treated with SALP for 20 min after virus inoculation. As controls, cells were either treated with PBS (control 1), rhodamine-conjugated PEP 19-2.5 (control 2) or infected with pH1N1 only. Cells were washed several times with PBS after each treatment step to remove unspecific binding and then fixed with 4% paraformaldehyde (PFA) or ethanol, respectively. In order to visualize influenza virus receptor components, fixed cells were incubated with 5 μg/ml FITC labeled *Sambucus nigra* lectin (Vector Laboratories) which preferentially recognize α2,6-linked N-Acetylneuraminic acids for 12 h at 4 °C. Later, cells were washed with PBS to remove unspecific binding. For virus staining, cells were incubated with anti-pH1N1 serum for 2 h at 37 °C. Again, cells were washed repeatedly with PBS and incubated with the secondary antibody anti-mouse-Alexa Fluor® 647 (1:500 diluted in PBS) for 20 min at 37 °C and embedded in Mowiol. Viral attachment to the cell surface was analyzed using a confocal microscope (Nikon C2+). The modification of 3D reconstructed Z-stacks was performed with the Imaris 7.6.1 (Bitplane) program. All experiments were performed at least two independent times.

To study viral entry, A549 cells were seeded to 80% confluency and infected with H7N7 influenza virus at an MOI of 25 to detect incoming NP at 2 h post infection (p.i.). Subsequently, cells were treated as described above. As controls, infected cells were either treated with PBS (control 1), PEP 19-CP (control 2) or untreated and uninfected (mock). Cells were fixed with 4% PFA and permeabilized with 0.3% Triton X. Next, cells were incubated with the primary mouse anti-NP antibody (1:750 diluted in PBS) for 2 h at room temperature. Anti-mouse Alexa Fluor® 488 (1:1,000 diluted in PBS) was used as secondary antibody. Images were taken using a confocal microscope (Nikon C2+).

2.8. Western blot analysis

To analyze the effect of impaired virus attachment on viral replication, A549 cells were infected with H7N7 influenza virus at an MOI of 1.0 to detect newly synthesized nucleoprotein (NP) at 6 h p.i. Treatment with PEP 19-2.5 (20 μg/ml diluted in PBS) was analyzed again in three different approaches: (i) pre-treatment, (ii) co-treatment and (iii) post-treatment as described above. As controls, infected cells were either treated with PBS (control 1), PEP 19-CP (control 2) or untreated and uninfected (mock). Next, cells were lysed with lysis buffer (50 mM HEPES (pH 8.0), 200 mM NaCl, 0.5% Igepal, 25% glycerol, 1 mM PMSF, 0.07 μl/ml β-mercaptoethanol, 1× HALT™ Protease and Phosphatase Inhibitor Cocktail (Pierce/Thermo Scientific), and 1× EDTA Solution (Pierce/Thermo Scientific)) and Western blot analysis was performed as described before (Hudjetz and Gabriel, 2012). Protein quantification was performed using Bioimager Image Quant LAS 4000. Levels of viral proteins (NP and PB2) of infected and PBS treated controls were set 100% and compared to SALP treated settings. All experiments were performed at least two to three independent times.

2.9. Transmission electron microscopy

To confirm virus binding on cell surfaces and its impairment by SALP, transmission electron microscopy (TEM) was performed. Therefore, A549 cells were seeded onto 35 mm culture dishes with imprinted grids (ibidi GmbH) to 40% confluency, treated with Rhodamine-conjugated PEP 19-2.5 (20 μg/ml diluted in PBS) for 20 min at room temperature and then incubated with 10^9 p.f.u. of H7N7 virus for 30 min at 4 °C to allow virus attachment. Cells either treated with PBS (control 1), Rhodamine-conjugated PEP 19-2.5 (control 2) or incubated with virus only served as controls. Cells were fixed directly after viral attachment with 2,5% glutaraldehyde (diluted in PBS) for 30 min at room temperature. Subsequently, cells were washed with PBS, stained with 1% OsO_4 in PBS for 30 min, washed with ddH_2O, treated with 0.5% gallic acid in PBS/ ddH_2O (1:1) and washed with 1% Na_2SO_4 in PBS/ddH_2O (1:1). The samples were gradually dehydrated with ethanol and embedded in Epon resin for sectioning. Single parts of the culture dishes were stamped out and sectioned horizontally. Ultrathin sections (50 nm) were prepared using an Ultracut Microtome (Reichert Jung). All sections were counterstained with 2% uranyl acetate and lead citrate. Electron micrographs were obtained with an OSIS Veleta CCD Camera attached to a FEI Technai G 20 Twin transmission electron microscope (FEI, Eindhoven, The Netherlands) at 80 kV.

2.10. Plaque assay

To assess the potential of SALPs to inhibit virus replication, we have used plaque assays on MDCK cells as described before (Gabriel et al., 2008). Briefly, MDCK cells were seeded into 6-well plates up to 80–90% confluency. Cells were co-treated with an inoculum containing serial 10-fold dilutions of the H7N7, H3N2 or pH1N1 influenza virus strains and the respective SALP (20 or 50 μg/ml). As controls, infected cells were either treated with PBS (control 1) or PEP 19-CP (control 2). After 30 min incubation at 37 °C, cells were covered with an avicel-overlay in the corresponding cell culture medium for further 2 days at 37 °C. Plaque forming units (p.f.u.) of individually treated groups and controls were determined. Viral titers obtained from infected and PBS treated control groups were set 100% and compared to the SALP treated groups. All experiments were performed at least four independent times.

2.11. Multicycle virus replication

To assess whether SALPs are able to inhibit virus replication in a post-treatment setting, multicycle growth kinetics were performed. Briefly, A549 cells were seeded into 6-well plates up to 90% confluency. Cells were infected with the H7N7 influenza virus strain at an MOI of 10^{-4}. After 1 h incubation at 37 °C, the inoculum was removed and cells were washed once with PBS. Subsequently, cells were covered with cell culture medium containing SALP PEP 19-2.5 (20 or 50 μg/ml). The supernatant was again replaced 24 h p.i. with fresh cell culture medium containing PEP 19-2.5. Supernatant was collected at three time points (1, 24 and 48 h p.i.) and viral titers were determined by plaque assay as described above. Viral titers obtained from infected and PBS treated control groups were set 100% and compared to those treated with PEP 19-2.5 or control peptide PEP 19-CP. Experiments were performed twice in duplicate.

2.12. Animal experiments

Animal experiments were performed according to the guidelines of the German animal protection law. All animal protocols were approved by the relevant German authority. BALB/c mice were bred and housed at the animal facility of the Heinrich Pette Institute, Leibniz Institute for Experimental Virology or obtained from Charles River Laboratories. Six to eight week old mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg).

To determine the toxicity of SALPs *in vivo*, anesthetized BALB/c mice were either treated intranasally once with 5 μg ($n = 10$), 20 μg ($n = 5$), 50 μg ($n = 9$) or 100 μg ($n = 10$) of PEP 19-2.5 or treated once a day for 7 days with 5 μg ($n = 5$) of PEP 19-2.5. Animals of control groups received PBS ($n = 10$) or SALP PEP 19-CP ($n = 10$). Body weight was monitored for 14 days. Two animals of the groups inoculated with 5 μg of PEP 19-2.5, PBS or the control peptide PEP 19-CP were euthanized 3 days post treatment and lungs were removed for histochemical staining with hematoxylin and eosin as described before (Otte et al., 2011). All images were taken with a light microscope (Zeiss Axioplan2 equipped with a Jenoptik C12 camera).

To determine the distribution of SALPs in the respiratory tract, BALB/c mice were intranasally inoculated with 5 μg rhodamine-conjugated PEP 19-2.5. Control animals received PBS. After 15, 30 or 45 min treatment, mice were euthanized and organs (trachea and lung) were removed for histological analyses. Cellular DNA was stained with DAPI. All images were taken with a fluorescence microscope (Nikon NI equipped with a Nikon DS-Fi2 camera).

To evaluate SALP efficacy upon co-treatment, anesthetized BALB/c mice ($n = 16$) were intranasally (i.n.) inoculated with a volume of 50 μl containing 10^4 p.f.u. of the mouse-adapted H7N7 virus (~15-fold LD_{50}) and 5 μg of the SALP PEP 19-2.5. Control groups received PBS ($n = 16$) or PEP 19-CP only ($n = 16$). Body weight and survival were monitored for 14 days. Lungs of two to three animals per group were harvested on days 3 and 6 p.i. and viral titers were determined by plaque assay as described before.

To evaluate SALP efficacy upon post-treatment, anesthetized BALB/c mice ($n = 9$) were i.n. inoculated with 2.3×10^3 p.f.u. of the mouse-adapted H7N7 virus (~5-fold LD_{50}) in a volume of 50 μl diluted in PBS. First i.n. treatment with 5 μg of PEP 19-2.5 was performed 30 min p.i. and continued once daily for further 6 days after isoflurane narcosis. Virus infected control groups were treated either i.n. with PBS (control 1; $n = 8$) or oral with 100 mg/kg Oseltamivir (Tamiflu®) ($n = 11$). Body weight and survival were monitored for 14 days. Lungs of three animals per group were harvested for immunohistochemical analysis on day 5 p.i. Images were taken with light microscope (Nikon NI equipped with a Nikon DS-Fi2 camera).

2.13. Immunohistochemistry

Murine lungs were formalin-fixed, paraffin-embedded and sectioned as described previously (Otte et al., 2011). Viral antigens were stained using a rabbit anti-H7N1 serum, a biotin-conjugated anti-rabbit secondary antibody and the ZytoChemPlus (HRP) Broad Spectrum (DAB) Kit (Zytomed) according to the manufacturer's instructions. Tissues were counterstained with hematoxylin. Images were taken with a light microscope (Zeiss Axioplan2 equipped with a Jenoptik C12 camera or Nikon NI equipped with a Nikon DS-Fi2 camera).

2.14. Statistics

Mean values and standard deviation (SD) were calculated and the statistical significance was assessed by Student's-*t*-test for unpaired data. For animal survival studies, the Gehan–Breslow–Wilcoxon test was used. Statistical significance was defined as $p < 0.01$ ($^{*}p < 0.01$, $^{**}p < 0.001$).

3. Results

3.1. SALPs bind to N-Acetylneuraminic acids

SALPs were reported to inhibit HIV, HCV and HSV cellular entry by interacting with heparan sulfate which serves as a co-receptor molecule for these viruses (Krepstakies et al., 2012). To evaluate whether SALPs are also able to bind N-Acetylneuraminic acids as major components of the influenza virus receptor, we performed an isothermal titration calorimetry assay. SALPs were titrated against N-Acetylneuraminic acids and the enthalpy change, which occurs upon direct interaction of the components, was measured. All SALP variants were able to bind to N-Acetylneuraminic acids in a strong exothermic reaction in contrast to the control peptide (PEP 19-CP) (Fig. 1A). The strongest exothermic reaction was measured for PEP 19-2.5, followed by PEP 19-8D and PEP 19-4 (Fig. 1B–D). Furthermore, saturation of N-Acetylneuraminic acids was observed at a molar ratio of [Peptide]:[N-Acetylneuraminic acid] = 5–6 for PEP 19-2.5 and 6–8 for PEP 19-8D, whereas PEP 19-4 displayed saturation at a higher molar ratio of 11–12 (Fig. 1). These findings demonstrate that SALPs have the potential to directly and specifically bind to N-Acetylneuraminic acids with PEP 19-2.5 showing the highest affinity.

3.2. SALPs inhibit influenza virus hemagglutination

To analyze whether SALP binding to influenza virus receptor molecules might affect virus attachment on erythrocytes which abundantly express N-Acetylneuraminic acids on their surface, we performed hemagglutination assays. Here, 100% hemagglutination of erythrocytes was observed with H7N7, H3N2 and pH1N1 influenza viruses in the absence of SALPs in contrast to controls where erythrocytes readily sedimented resulting in 0% hemagglutination in the absence of virus (Fig. 2). SALPs were able to inhibit the hemagglutination of all three virus subtypes, albeit at various degrees, compared to erythrocytes treated with PBS or the control peptide PEP 19-CP only. SALPs PEP 19-2.5, PEP 19-4 and PEP 19-8D inhibited virus hemagglutination up to 75% depending on virus subtype and dilution. Here, we show that SALPs, especially PEP 19-2.5 and PEP 19-8D, are able to inhibit influenza virus hemagglutination of H7, H3 and H1 subtypes suggesting that they might interfere with virus adsorption to erythrocytes.

3.3. SALP PEP 19-2.5 inhibits influenza virus cellular attachment

In order to analyze whether inhibition of hemagglutination by SALPs also results in altered virus attachment to cell surfaces, we performed indirect immunofluorescence assays. Therefore, A549 human lung cells were inoculated with pH1N1 virus at 4 °C and cellular adsorption was detected 30 min later. The efficacy of SALP PEP 19-2.5 which showed highest receptor binding affinity and hemagglutination inhibition activity was evaluated in either a pre-, co- or post-treatment approach. In the pre-treatment approach, cells were treated with PEP 19-2.5 before virus inoculation, whereas in the co-treatment setting virus inoculation and SALP treatment occurred simultaneously. The post-treatment approach represents SALP treatment after virus incubation. Uninfected cells treated with either PBS (control 1) or PEP 19-2.5 (control 2) only served as controls. Further, all cells were stained against the most abundantly expressed influenza virus receptor component on A549 cells, α2,6-Neuraminic acid (α2,6-SA), with *S. nigra* lectin. PEP 19-2.5 treatment resulted in overall homogenous distribution on the

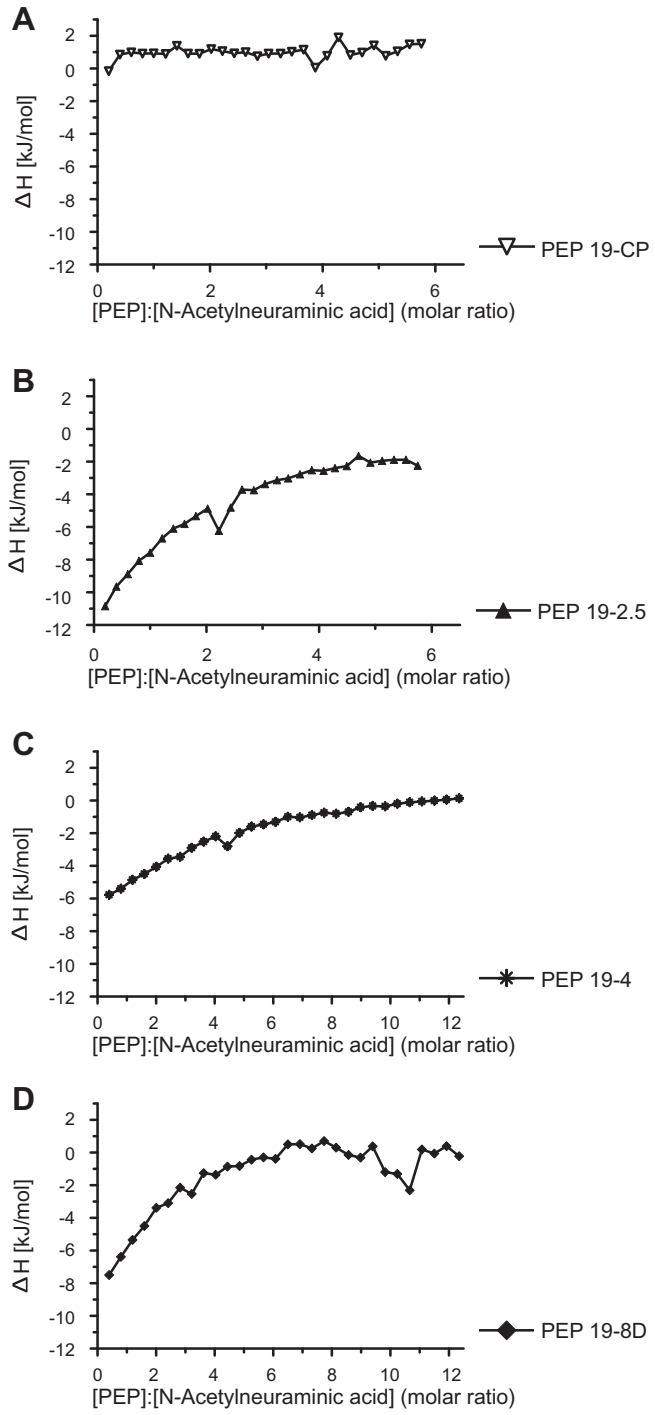

Fig. 1. Binding affinities of SALPs to N-Acetylneuraminic acids. For isothermal titration calorimetry, control peptide PEP 19-CP (5 mM) (A) or the individual SALPs PEP 19-2.5 (5 mM) (B), PEP 19-4 (10 mM) (C) or PEP 19-8D (10 mM) and (D) were titrated against N-Acetylneuraminic acid (0.05 mM). The enthalpy was measured by MSC isothermal titration calorimeter (Microcal Inc.). Titration of the peptides was stopped when saturation was observed at a molar ratio of (Peptide):(N-Acetylneuraminic acid) = 6 (A, B) or 12 (C, D). Here, a representative of at least two independent experiments is shown.

cell surface of uninfected and infected cells. Virus antigen detection was considerably reduced in the pre- and co-treatment setting similar to uninfected controls. In the post-treatment approach, virus antigen detection was only slightly reduced compared to infected but non-treated cells (Fig. 3). Interestingly, α2,6-SA detection was reduced in infected and PEP 19-2.5 pre-treated cells similar to SALP-treated but uninfected controls. This might suggest that there is a competition between PEP 19-2.5 and lectins for α2,6-SA binding. However, α2,6-SA detection was not affected in cells co-treated with PEP 19-2.5 albeit virus detection was strongly reduced. This might further suggest that PEP 19-2.5 might additionally bind to virus particles, thereby blocking virus attachment. Taken together, PEP 19-2.5 binding to N-Acetylneuraminic acid strongly correlates with significantly reduced viral attachment on cells in a pre- and co-treatment approach. However, PEP 19-2.5 might also impair virus cellular attachment by additionally binding to virus particles and hindering cellular adsorption.

3.4. SALP PEP 19-2.5 binds to influenza virus particles

To further elaborate whether SALP PEP 19-2.5 might block virus attachment to cells by an additional mechanism besides binding to influenza virus receptor components, namely by directly binding to virus particles, we performed transmission electron microscopy (TEM). TEM analysis revealed visible PEP 19-2.5 aggregates in uninfected controls (Fig. 4). It should be noted that single SALP molecules would not be visible using this technique. As expected, H7N7 virus particles were readily visible in infected but untreated cells. Interestingly, in treated cells, virus particles were predominantly attached to PEP 19-2.5 aggregates. These findings suggest that PEP 19-2.5 blocks influenza virus cellular attachment by two distinct mechanisms. On one hand it directly binds to influenza virus receptor components and on the other hand it additionally binds to virus particles.

3.5. SALP PEP 19-2.5 inhibits influenza virus cell entry and replication

We further analyzed whether reduced influenza virus attachment to cells upon SALP treatment results in impaired virus entry and replication. To analyze virus entry, immunofluorescence analysis was performed using again pre-, co- and post-treatment approaches with PEP 19-2.5 upon H7N7 influenza virus infection. In contrast to the attachment studies performed above, here, the cells were permeabilized 2 h p.i. and stained for viral nucleoprotein (NP) as a marker for virus entry. Viral NP could be abundantly detected in infected cells either treated with PBS or the control peptide (PEP 19-CP) (Fig. 5A). NP detection was significantly reduced in cells treated with PEP 19-2.5. Strongest NP reduction was observed in the pre- and co-treatment approach followed by the post-treatment settings. This suggests that reduced virus attachment to cells described above results in reduced virus entry. Next, Western blot analysis was performed in a similar setting 6 h p.i. to allow single-cycle virus replication. Consistently, virus replication detected using the viral polymerase subunit PB2 and NP were almost completely abolished in the pre- and co-treatment setting and reduced by more than 50% in the post-treatment approach (Fig. 5B and C). Thus, PEP 19-2.5 inhibits virus attachment thereby leading to impaired virus entry and replication.

3.6. SALP PEP 19-2.5 inhibits replication of several influenza virus subtypes

Next we addressed whether PEP 19-2.5 treatment would reduce replication of various influenza virus subtypes. Therefore, we infected MDCK cells with H7N7, H3N2 or pH1N1 subtypes and co-treated with PEP 19-2.5 and other SALPs described above. The viral titer of infected and PBS treated cells was set to 100%. SALPs PEP 19-8D and PEP 19-2.5 displayed the strongest inhibition of H7N7, H3N2 and pH1N1 virus replication compared to controls which were either treated with PBS or the control peptide (PEP 19-CP) by 1–4 orders of magnitude given at a dose of

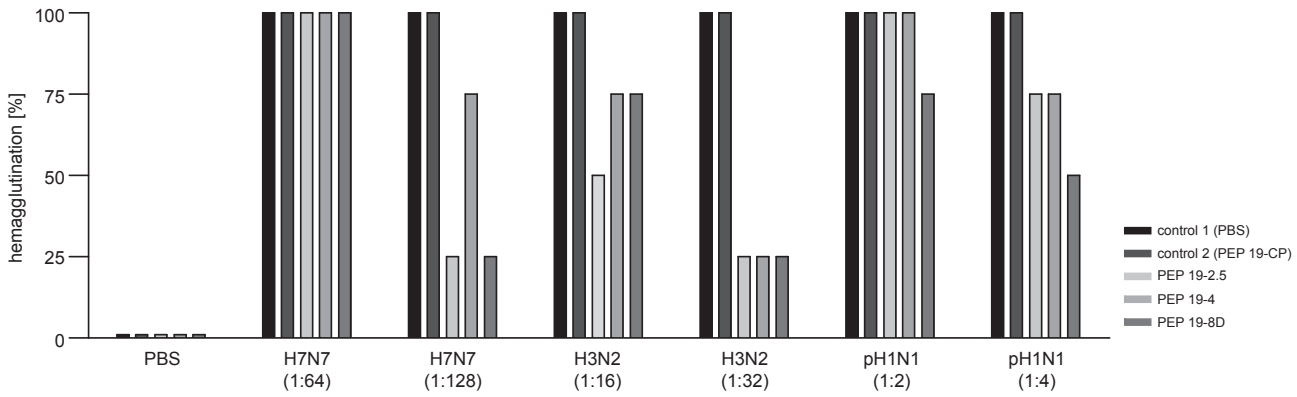

Fig. 2. Efficacy of SALPs to inhibit viral hemagglutination to chicken erythrocytes. Chicken erythrocytes were incubated with H7N7, H3N2 or pH1N1 influenza viruses containing 5 μg/ml of the indicated SALP (PEP 19-2.5, PEP 19-4, PEP 19-8D) or as controls, with PBS (control 1) or the non-positive charged control peptide PEP 19-CP (control 2). Hemagglutination mediated by H7, H3 and H1 virus subtypes at two different dilutions of the corresponding virus stock (for H7N7, 1:62 and 1:128; for H3N2, 1:16 and 1:32; for pH1N1, 1:2 and 1:4 dilution in PBS) was assessed 45 min after incubation on ice. The grade of viral hemagglutination in the absence of SALPs was set as 100%. Complete failure to hemagglutinate erythrocytes in the absence of virus was set as 0%. Gradual inhibition of hemagglutination in the presence of SALPs and virus is described in percentage. Here, a representative of at least two independent experiments is shown.

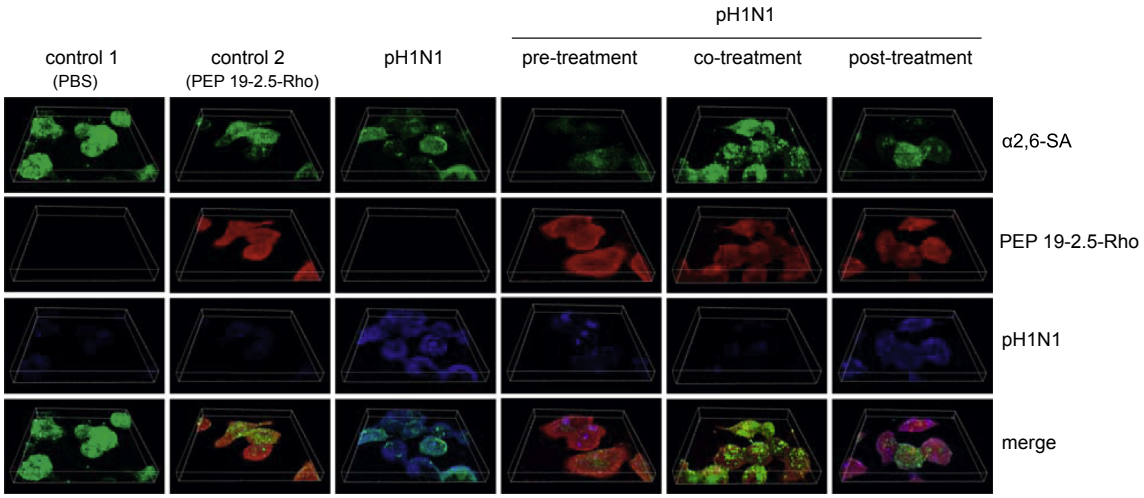

Fig. 3. Efficacy of SALP PEP 19-2.5 to inhibit viral attachment to human cells. A549 cells were inoculated with 10^5 p.f.u. of pH1N1 influenza virus for 30 min at 4 °C. Rhodamine-conjugated PEP 19-2.5 (red) treatment (20 μg/ml) was performed either prior viral inoculation (pre-treatment), simultaneously (co-treatment) or after viral inoculation (post-treatment). Staining of α2,6-linked N-Acetylneuraminic acids (α2,6-SA) was performed with FITC-labeled *Sambucus nigra* lectin (green). Viral antigen was stained using an anti-pH1N1 mouse serum and anti-mouse-Alexa Fluor® 647 as a secondary antibody (blue). Cells either treated with PBS (control 1), SALP PEP 19-2.5 (control 2) or pH1N1 served as controls. Images were taken using a confocal microscope. The 3D reconstructed Z-stacks were modified with the Imaris 7.6.1 program. Here, a representative of at least two independent experiments is shown.

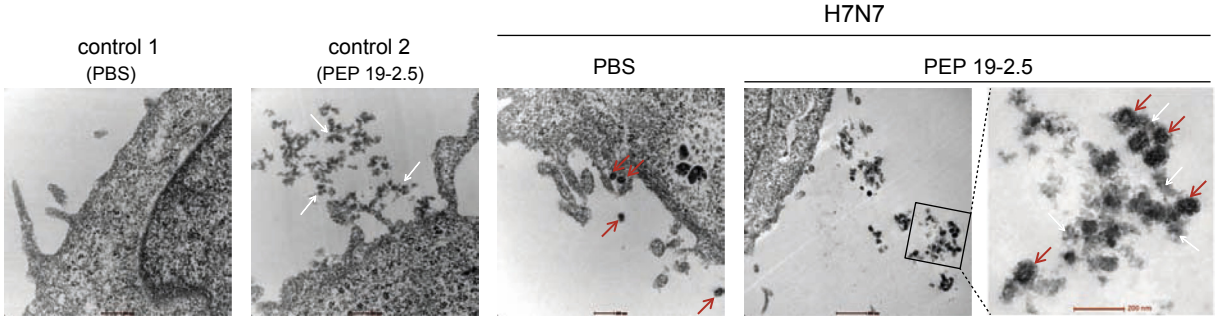

Fig. 4. SALP PEP 19-2.5 attachment to virus particles. A549 cells were treated with Rhodamine-conjugated PEP 19-2.5 (20 μg/ml) and then inoculated with 10^9 p.f.u. H7N7 influenza virus for 30 min at 4 °C. Cells either treated with PBS (control 1), SALP PEP 19-2.5 (control 2) or H7N7 served as controls. After treatment with 0.5% gallic acid and staining with 1% OsO_4, samples were dehydrated and sectioned. Images of counterstained ultrathin sections were taken using the FEI Technai G 20 Twin transmission electron microscope. SALP aggregates are indicated by white arrows. Viral particles are indicated by red arrows. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

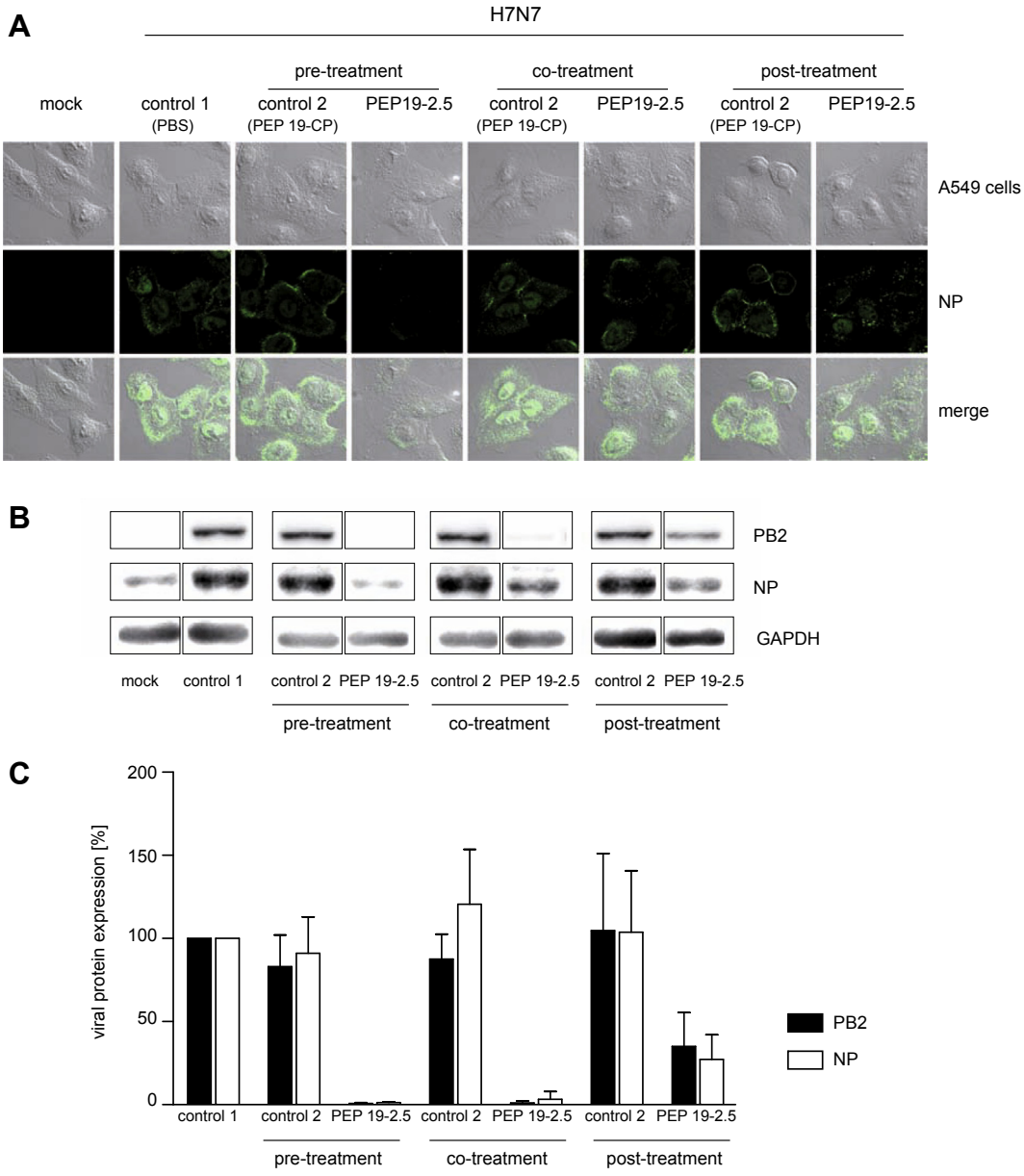

Fig. 5. SALP PEP 19-2.5 mediated inhibition of viral entry and replication in human cells. A549 cells were inoculated with H7N7 influenza virus at MOI 25 (A) or MOI 1 (B and C) for 30 min at 4 °C. Treatment with SALP PEP 19-2.5 (20 μg/ml) was performed either prior viral inoculation (pre-treatment), simultaneously (co-treatment) or after viral inoculation (post-treatment). As controls, cells were either untreated and uninfected (Mock) or infected and treated with PBS (control 1) or the control peptide PEP 19-CP (control 2). Viral entry was analyzed by detection of viral NP (green) at 2 h p.i. using a confocal microscope (A). Viral protein synthesis was determined 6 h p.i. by Western blot analysis using specific antibodies against NP and PB2 (B). Quantification of viral protein levels was performed using Bioimager Image Quant LAS 4000. Background signals of mock cells were subtracted and viral protein levels were normalized against the internal standard protein GAPDH. The viral proteins of infected and PBS treated control groups were set 100%. (C). Mean values and standard deviations (SD) were calculated from at least two independent experiments and representatives thereof are shown.

20 μg/ml ($p < 0.001$) (Fig. 6A, C and E). The inhibitory potential of SALPs was further increased at a concentration of 50 μg/ml ($p < 0.001$) (Fig. 6B, D and F). However, all tested SALPs were most effective against H7N7 (Fig. 6A and B) compared to H3N2 (Fig. 6C and D) or pH1N1 virus (Fig. 6E and F) replication. Treatment of cells with the individual SALPs resulted in no or little cytotoxicity even at higher concentrations (see Supplementary Fig. 1). These data demonstrate that SALP variants are able to inhibit influenza virus replication of H7, H3 and H1 subtypes up to 4 orders of magnitude in a dose dependent manner. However, SALP PEP 19-2.5 and PEP 19-8D show the strongest inhibitory effect at non-toxic concentrations.

3.7. SALP PEP 19-2.5 co-treatment protects against lethal H7N7 influenza virus infection in mice

To study whether SALPs are also able to inhibit lethal influenza virus replication in mice, we have assessed the antiviral potential of PEP 19-2.5, as the most promising candidate of our *in vitro* studies, in a lethal H7N7 mouse infection model. First, we have established the dose regimen for PEP 19-2.5 in mice where no signs of toxicity could be observed (Supplementary Fig. 4). Next, mice were intranasally treated with 5 μg of PEP 19-2.5 or the control peptide (PEP 19-CP) where no weight loss (Fig. 7A), pathological alterations in the lung (Fig. 7B) or any other signs of toxicity could be observed. Fur-

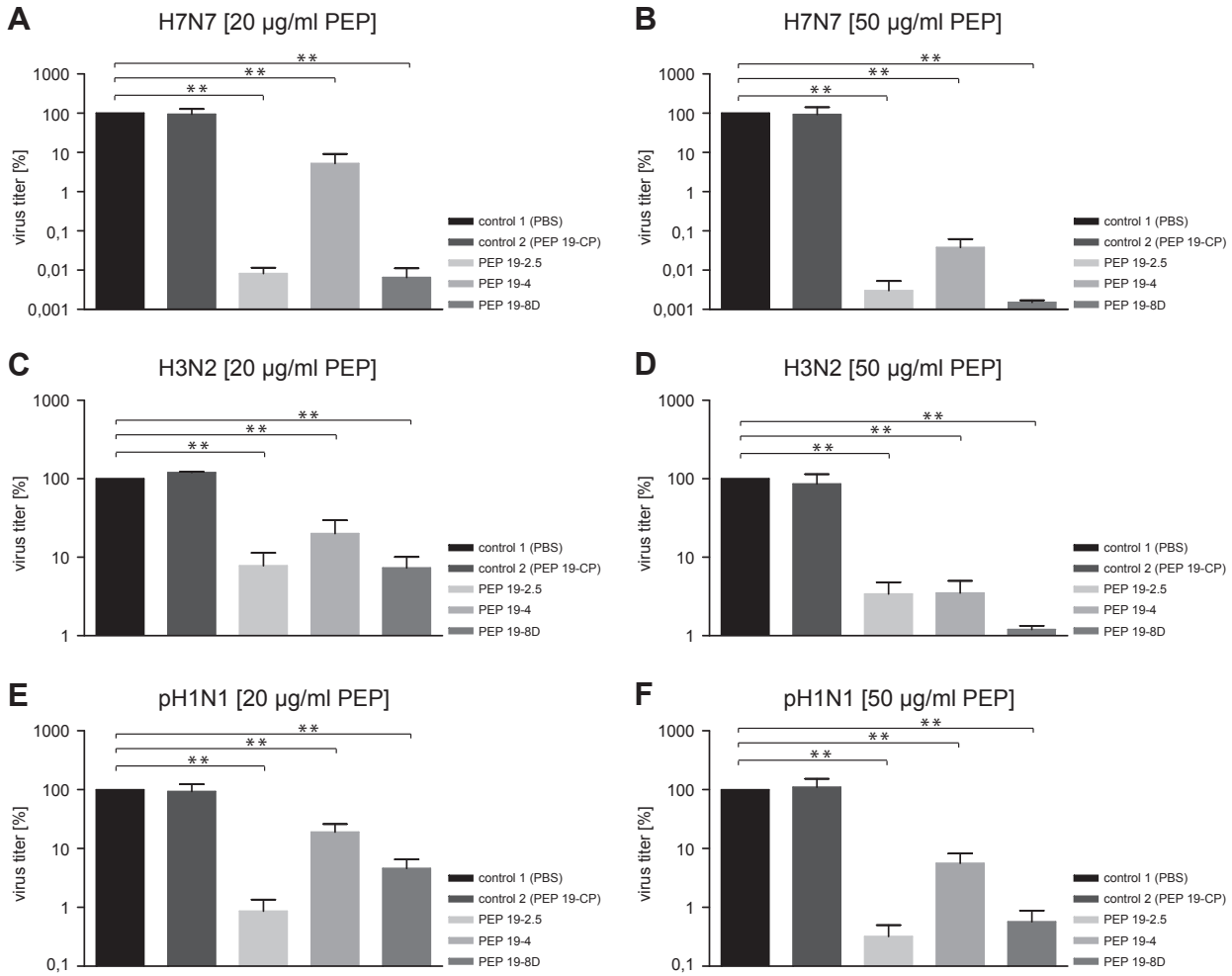

Fig. 6. Antiviral effect of SALPs on influenza virus replication. MDCK cells were infected with either H7N7 (A and B), H3N2 (C and D) or pH1N1 influenza viruses (E and F) and co-treated with either 20 µg/ml (A, C and E) or 50 µg/ml (B, D and F) SALPs. Virus titers were determined by plaque assay on MDCK cells as described previously (Gabriel et al., 2008). Viral titers of infected but PBS treated control groups (control 1) were set 100%. As a further control, cells were treated with the control peptide PEP 19-CP and infected as described above (control 2). Mean values and standard deviation (SD) were determined from at least four independent experiments. The statistical significance was assessed by the Student's-*t*-test ($^{*}p < 0.01$, $^{**}p < 0.001$).

thermore, intranasally administered rhodamine-conjugated PEP 19-2.5 was readily detected on epithelial cells of the murine respiratory tract, such as the lung and trachea suggesting that PEP 19-2.5 reaches the area of designated target cells where influenza virus attachment and replication occurs (Fig. 7C). We then assessed the inhibitory effect of SALP PEP 19-2.5 by infecting BALB/c mice with a high H7N7 virus dose corresponding to $15 \times LD_{50}$. Animals were co-treated either with PEP 19-2.5 or the control peptide (PEP 19-CP) (Fig. 8). All PEP 19-2.5 co-treated animals survived an otherwise 100% lethal infection in mice without showing any significant weight loss. In contrast, all mice co-treated with PBS or PEP 19-CP as controls significantly lost weight and succumbed to infection on days 7 and 8, respectively ($p < 0.001$) (Fig. 8A and B). Furthermore, virus replication in the lung was almost completely abolished in PEP 19-2.5 treated animals with only 1 out of 3 animals showing strongly reduced virus titers on day 6 p.i. compared to high virus titers in the control groups on days 3 ($p < 0.001$) and 6 p.i. ($p < 0.05$) (Fig. 8C). Taken together, these data show that co-treatment with SALP PEP 19-2.5 inhibits influenza virus replication and prevents from lethal infection in mice.

4. Discussion

Currently available drugs for treatment of influenza virus infections are directed against the M2 ion channel and the neuraminidase (NA). Some influenza virus strains already developed resistance against these drugs by specific mutations in their respective M2 or NA genes (Hayden and de Jong, 2011). Therefore, efficiency of these drugs is limited and the development of novel antiviral strategies has become an emerging need.

SALPs have been first described by Gutsmann and colleagues as multifunctional peptides with antimicrobial and anti-inflammatory activities against Gram-positive and -negative bacteria (Gutsmann et al., 2010; Kaconis et al., 2011; Schuerholz et al., 2012). Recently, an additional broad-spectrum antiviral activity of SALPs against some enveloped viruses such as HIV, HBV, HCV and HSV have been described. There, it was shown that SALPs can directly bind to heparan sulfates which act as co-receptors for various human pathogenic viruses and thereby inhibit virus attachment and entry (Krepstakies et al., 2012).

In the present study, we report that SALPs are able to inhibit influenza virus replication of various influenza virus subtypes (H7, H3 and H1) by preventing virus attachment to host cells *in vitro* and *in vivo*.

We could show that SALPs were able to directly bind to N-Acetylneuraminic acids as major components of the influenza virus receptor with PEP 19-2.5 showing the highest binding affinity. The polycationic nature of SALPs has been described to mediate reduced viral replication for a range of different viruses by overall

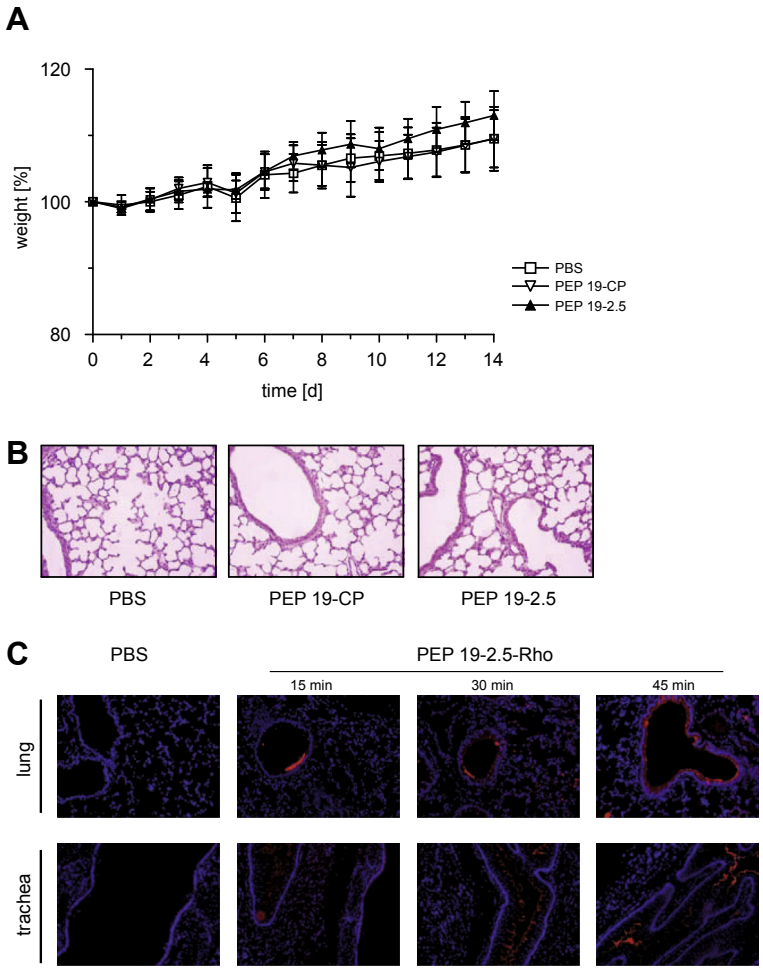

Fig. 7. Toxicity and distribution of SALP PEP 19-2.5 in the respiratory tract of BALB/c mice. BALB/c mice ($n = 10$) were administered intranasally with 5 μg of PEP 19-2.5. Control groups were treated with PBS ($n = 10$) or 5 μg of the control peptide PEP 19-CP ($n = 10$). Body weight (A) was monitored for 14 days after treatment. Lung tissues of two animals per group were obtained three days after treatment and counterstained with hematoxylin and eosin (B). To analyze the distribution of SALPs in the mammalian airway, mice were treated intranasally with 5 μg of rhodamine-conjugated PEP19-2.5 (PEP 19-2.5-Rho) or received PBS as a control. Lung and trachea were removed 15, 30 or 45 min after peptide treatment and tissue sections were stained with DAPI. Images were taken with a light microscope (B) or a fluorescence microscope (C). Mean averages with SD are shown.

positive charges and the equal distribution thereof over the entire peptide rather than peptide sequence and amphiphilicity itself (Krepstakies et al., 2012). This is consistent with our findings described here since the antiviral activity was not observed when a control peptide with non-positive charged amino acid sequences was used suggesting that positively charged peptides are crucial for the antiviral activity of SALPs.

Consistently, SALPs were able to efficiently inhibit viral hemagglutination to chicken erythrocytes suggesting that agglutination is inhibited by competitive binding to N-Acetylneuraminic acids on erythrocytes. Increased SALP concentrations only partially enhanced their ability to inhibit hemagglutination suggesting that lower SALP concentrations are sufficient to block receptor recognition. However, at high SALP concentrations we additionally observed that some SALPs were able to agglutinate red blood cells at various degrees even in the absence of virus (Supplementary Fig. 2). This was consistent with our TEM analysis where SALPs formed aggregates. These data suggest that potential multiple binding sites on SALPs would allow them additionally to interact with each other. Thus, providing an explanation how SALPs would be able to agglutinate erythrocytes. However, PEP 19-2.5 was able to block virus attachment on human lung cells and thereby inhibit virus entry and replication of pH1N1 and H7N7 influenza viruses. In all experimental settings pre- and co-treatment approaches were more effective than post-treatment with PEP 19-2.5.

Immunofluorescence and TEM data suggest that virus attachment to cells by PEP 19-2.5 is impaired by two mechanisms. PEP 19-2.5 is able to directly interact with N-Acetylneuraminic acids and additionally to bind to virus particles. Influenza viruses are enveloped viruses with two major glycoproteins embedded into the viral membrane, the hemagglutinin and the neuraminidase. It is unclear whether PEP19-2.5 might bind to any of the viral surface proteins or interact with the viral membrane which derives from the host cell by virus budding. Clearly, future studies are needed to address how PEP 19-2.5 might attach to influenza virus particles. However, it is tempting to speculate that SALPs might rather bind to various negatively charged membrane components (such as heparan sulphate and N-Acetylneuraminic acid) than complex glycosylated viral proteins due to their positively charged nature. Thus, virus attachment and entry into host cells is blocked by masking the viral receptor on one hand and preventing newly released virus particles from secondary infection on the other hand.

SALP PEP 19-2.5 was additionally highly effective in inhibiting viral replication of H7, H3 and H1 subtypes with a reduction in virus titers up to 4 orders of magnitude upon co-treatment. This is particularly notable since the H7N7 virus strain used in this

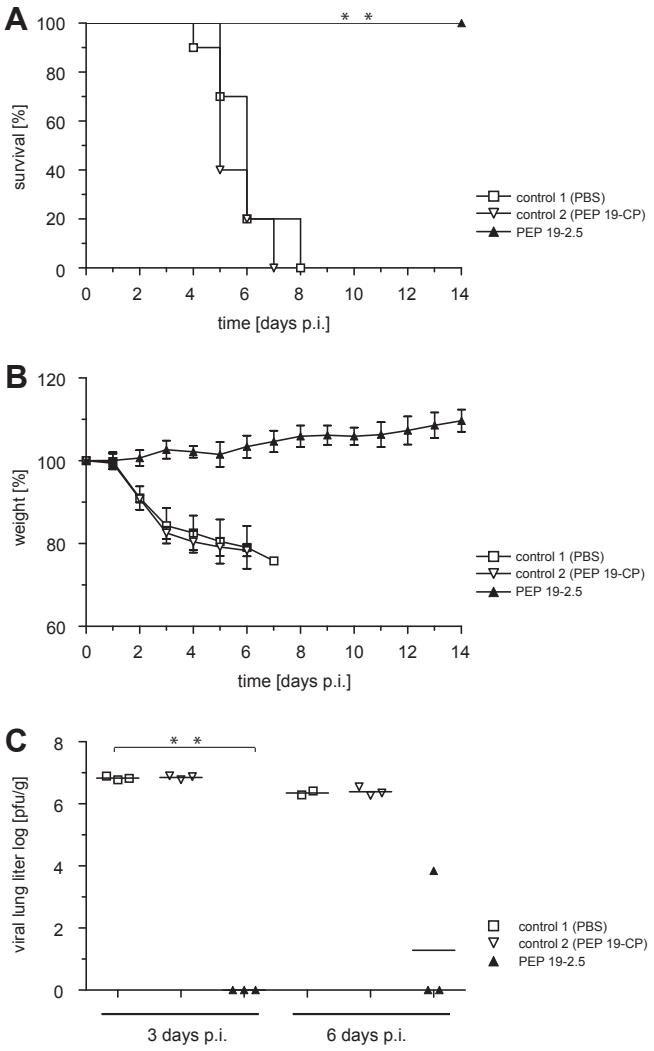

Fig. 8. Pathogenicity of H7N7 infected BALB/c mice upon SALP PEP 19-2.5 treatment. BALB/c mice ($n = 16$) were intranasally inoculated with 10^4 p.f.u. of the H7N7 influenza virus (~ 15-fold LD_{50}) combined with 5 μg of PEP 19-2.5. Control groups were infected with 10^4 p.f.u. of the H7N7 virus and treated either with PBS (control 1) ($n = 16$) or 5 μg of the control peptide PEP 19-CP (control 2) ($n = 16$). Survivals (A) and body weights (B) were monitored for 14 days p.i. Lungs of two to three animals per group were harvested on days 3 and 6 p.i. and viral titers were detected by plaque assays (C). Mean values and SD were determined. The statistical significance was assessed by the Gehan–Breslow–Wilcoxon test for the survival date and Student's *t*-test for the virus titers ($^*p < 0.01$, $^{**}p < 0.001$).

study possesses very high replication rates and is highly virulent in mice (Gabriel et al., 2005). Upon therapeutic treatment, viral replication was reduced up to 80% at lower and up to 95% at higher non-toxic SALP concentrations (Supplementary Fig. 3). Furthermore, SALPs showed differential antiviral activities towards human (pH1N1, H3N2) and avian (H7N7) influenza viruses. Inhibition of viral agglutination to chicken erythrocytes which predominantly express α2,3-SA and are preferably recognized by avian viruses was most effective for H7N7 and H3N2 virus subtypes but less efficient against pH1N1. Both human isolates, H1 and H3 preferentially bind to α2,6-SA receptor components while the avian H7 virus prefers α2,3-SA. Moreover, virus attachment to human lung cells was impaired in pH1N1 infected cells upon PEP 19-2.5 pretreatment with masked α2,6-SA receptor components. Consistently, H7N7 virus entry was also very efficiently inhibited in human lung cells which abundantly express α2,6-SA receptors. Thus, there does not seem to be a correlation of SALP binding to α2,3- or α2,6-linked receptors and inhibition of virus attachment, entry and replication between avian and human influenza viruses. It should be noted that influenza virus recognition of cellular receptor molecules is more complex and cannot be reduced solely to α2,3- or α2,6-linked sialic acid components expressed on the cell surface. Future studies would be required to characterize SALP binding efficiency to influenza virus receptor components using e.g. glycan arrays expressing a variety of complex modified N-Acetylneuraminic acids (Stevens et al., 2006).

Remarkably, PEP 19-2.5 was able to protect treated mice from lethal influenza virus infection. All mice co-treated with a lethal mouse-adapted H7N7 influenza virus strain and SALP PEP 19-2.5 at non-toxic concentrations (Supplementary Fig. 4A) were protected against an otherwise 100% lethal infection. Furthermore, rhodamine-labeled SALPs could be detected on the surface of epithelial cells of the murine respiratory tract very early after treatment. Although it cannot be excluded that the label might affect SALP distribution on lung tissues itself, there is a strong correlation between SALP presence on epithelial cells and the almost complete inhibition of virus replication in the lungs of infected mice upon treatment. Thus, efficient delivery of SALPs into the respiratory tract seems to be crucial for blocking viral replication in the lung resulting in 100% survival of an otherwise 100% lethal infection in mice. Daily post-treatment of mice with PEP 19-2.5 at a non-toxic dose (Supplementary Fig. 4B) did not protect against lethal H7N7 influenza virus infection. However, lungs of post-treated mice were less infiltrated by mononuclear cells and alveoli destruction was strongly reduced compared to untreated animals (Supplementary Fig. 5). Oseltamivir treatment of H7N7 infected mice on the other hand led only to 20% survival accompanied by reduced viral antigen detection and inflammation in the lung. This is consistent with other studies where therapeutic oseltamivir treatment in mice is less efficient against highly pathogenic avian influenza virus strains compared to less virulent human strains (Smee et al., 2010; Takano et al., 2013). One should note that the highly virulent H7N7 strain used in this study does not harbour any of the previously described mutations in NA at amino acids positions 274, 292 and 294 linked to oseltamivir resistance (Baranovich et al., 2011; Moscona, 2009) However, the *in vivo* data obtained upon post-treatment evaluation of SALP PEP 19-2.5 efficacy are consistent with the *in vitro* data. While pre- and co-treatment with PEP 19-2.5 showed highest antiviral activity, post-treatment was less efficient. These findings suggest that further improvement of SALP design is required e.g. by further increasing its binding affinity and specificity for influenza virus receptor molecules as well as reducing its unspecific binding properties to achieve optimal SALP antiviral activity.

In summary, SALPs as small peptides are excellent candidates for the development of future antiviral strategies. Other promising antiviral approaches, such as RNA interference, inhaled sialidases, antibodies, small molecules etc. were evaluated before (Beigel and Bray, 2008; Clercq, 2012; Nicholls et al., 2013). The advantages of peptides for antiviral use comprise their small size allowing specific inhibition of protein–protein interactions, their low cytotoxicity in general as well as their immediate availability by peptide synthesis. It should also be noted that small peptides might also have some disadvantages, such as the high costs of peptide synthesis and potentially their stability limiting efficient storage for stockpiling purposes (Huther and Dietrich, 2007; Tan et al., 2012). However, the multiple advantages of small peptides have already been used successfully to develop peptide-based vaccines or inhibitors against viruses, e.g. HIV (ElSawy et al., 2012; Fox, 2007; Kang et al., 2011; Miller et al., 2011).

This study aimed to assess whether the broad-spectrum antimicrobial and antiviral activities of SALPs can be extended to influenza viruses known to pose a major pathogen responsible for respiratory infections and often bacterial co-infections in humans.

Clearly, future studies are needed to improve and evaluate the therapeutic potential of the SALPs in animal models e.g. where aerosolized antivirals could be directly delivered to the lungs by special inhalation tubes and nebulizers (Droebner et al., 2011) .

5. Conclusions

To conclude, our studies demonstrate that SALPs provide an excellent basis for future drug development against lethal influenza virus infections. Furthermore, SALPs might additionally provide a promising future strategy to combat several viral as well as viral/bacterial co-infections by targeting commonly used host cell proteins.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2014.01.015.

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