Published in final edited form as:

Biomaterials. 2013 May ; 34(15): 3846–3857. doi:10.1016/j.biomaterials.2013.01.026.

Antiviral peptide nanocomplexes as a potential antiviral therapeutic modality for HIV/HCV co-infection

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Abstract

It is estimated that 4 to 5 million people are currently co-infected with Human Immunodeficiency Virus (HIV) and Hepatitis C Virus (HCV). HIV/HCV co-infection is associated with unique health risks including increased hepatotoxicity of antiretrovirals, accelerated progression of HCV and liver diseases. The standard interferon-based therapy is effective only in about 50% of patients and often is associated with autoimmune and neuro-psychiatric complications. The treatment of co-infection (HIV/HCV) requires new strategic approaches. To this end, the formulations of an amphiphatic α -helical peptide, a positively charged analog of C5A peptide derived from the HCV NS5A protein, with a reported virocidal activity were prepared by electrostatic coupling with anionic poly(amino acid)-based block copolymers. The self-assembled antiviral peptide nanocomplexes (APN) were ca. 35 nm in size, stable at physiological pH and ionic strength, and retained *in vitro* antiviral activity against HCV and HIV. Moreover, incorporation of the peptide into APN attenuated its cytotoxicity associated with the positive charge. *In vivo* APN were able to decrease the viral load in mice transplanted with human lymphocytes and HIV-1-infected. Overall, these findings indicate the potential of these formulations for stabilization and delivery of antiviral peptides while maintaining their functional activity.

Keywords

amphiphatic α -helical peptide; anionic poly (amino acid)-based block copolymer; HIV-1; HCV; macrophages, human hepatoma cell line Huh7.5

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1. Introduction

In the United States and Europe approximately one-third of HIV-1 infected individuals are co-infected with HCV [1–4]. The prevalence of HCV co-infection is about 70 – 95% in current or former drug users and transfused hemophiliac patients, and became a leading cause of morbidity and mortality amongst this patient population [2, 5–7]. Both viruses potentiate each other's progression, complicate therapeutic application of antiretroviral drugs due to increased liver toxicity and interferon-based treatment for HCV, due to myelotoxicity, severe cognitive impairment and psychotic complications [1, 3, 8–16]. New therapeutic modalities are urgently required for treatment of HIV/HCV co-infection. Not many compounds are known to be effective antiviral (HCV) or antiretroviral (HIV) agents. The specific blockade of two viruses by one compound is precluded by several factors. There are diverse virus-susceptible host cells – macrophages, lymphocytes, and hepatocytes. The simultaneous drug targeting of multiple pathways of virus-host cell interactions and dissemination is also challenging. However, non-specific virocidal compounds could find their niche as antiviral/antiretrovirals and their inclusion in combination of therapeutics with different mechanisms of action could be beneficial. For HCV treatment antiviral peptide therapeutics potentially may reduce the time needed for achievement of stable virologic responses and be effective in IFN- α -non-responders [17–19].

A virocidal peptide derived from the membrane anchor domain of the HCV nonstructural protein NS5A (C5A) was found to be effective to inhibit both HIV [20] and HCV infections as well as infections with other human *Flaviviridae* members in cell culture systems [21]. The mechanism includes prevention of initiation of infection by destroying the virus and suppressing ongoing infections by blocking the cell-to-cell spread of the virus. It has been suggested that C5A recognizes cellular components of virus membranes most likely associated with the membrane lipid composition. Even demonstrating a low toxicity and immunogenicity [20, 21], the therapeutic *in vivo* application remains questionable due to sensitivity to proteolysis and immune activation properties [22]. A technology based on incorporation of proteins and polypeptides into polyion complexes with oppositely charged block copolymers, block ionomer complexes (BIC), can be used to deliver potent polypeptides while mediating the clinical delivery challenges. Proteins, peptides, and enzymes may be encapsulated in BIC to preserve activity and promote stability in the body [23–25]. When pH of the solution exceeds the isoelectric point, a protein becomes charged and can form protein-polyelectrolyte complexes with an oppositely charged block ionomer (Fig. 1A). Stability of the protein incorporated into BIC can be further improved by introducing hydrophobic groups to block copolymer or by cross-linking [25, 26]. The pH and salt sensitivity of BIC also provide a unique opportunity to control the triggered release of the protein [27]. Structure-activity relationship analysis of modified C5A (SWLRDIWDWICEVLSDFK), further abbreviated as p1, performed by Cheng and co-authors revealed that a cationic derivative of this peptide (SWLRRIRWICKVLSRKF), abbreviated as p41, with a net charge of +6 at pH 7, also displayed virocidal activity against HCV. The objective of this work was to characterize the broadness of antiviral properties of cationic peptide p41, to immobilize it into a peptide/polyion BIC using negatively charged block copolymers of poly(ethylene glycol) (PEG) and poly(amino acids), and to demonstrate *in vitro* and *in vivo* activity of an antiviral peptide nanocomplex (APN).

2. Materials and methods

2.1 Materials

Peptides SWLRDIWDWICEVLSDFK (p1), SWLRRIRWICKVLSRKF (p41) and p41-Cy5 were custom synthesized by AnaSpec (Fremont, CA). Block copolymers of methoxy poly(ethylene glycol)-*block*-poly(α,β -aspartic acid) (PEG-PLD_n) and methoxy-

poly(ethylene glycol)-*block*-poly(L-glutamic acid) (PEG-PLG_m) with PEG molecular mass of 5,000 Da and different length of anionic segments (n and m represent the degree of polymerization of PLD and PLE blocks) were obtained from Alamanda Polymers, Inc. (Madison, AL). The list of block copolymers used in this work and their molecular characteristics are presented in Table 1. For *in vitro* application peptides were dissolved in DMSO at 10mM concentrations and adjusted to the final concentrations of 2.5 μ M – 10 μ M by phosphate buffered saline (PBS) or culture medium.

2.2 Synthesis of APN

APN were prepared by mixing buffered solutions (phosphate buffer, 10 mM, pH 7, or PBS) of p41 peptide and anionic block copolymer at various compositions. The composition of the mixtures ($Z_{-/+}$) was calculated as a molar ratio of carboxylic groups in the copolymer to the amino groups in lysine and arginine residues of the peptide. As an example, the p41/PLD₂₀ APN at composition of $Z_{-/+}=1$ was prepared by mixing 5 μ l p41 (5 mg/ml) and 2.38 μ l PEG-PLD₂₀ (10 mg/ml) in the presence of buffer.

2.3 Dynamic Light Scattering (DLS)

Electrophoretic mobility and hydrodynamic diameters of APN were determined by DLS using Nano ZS Zetasizer (Malvern Instruments, UK) at a fixed 173° scattering angle. All measurements were performed in automatic mode at 25 °C. Software provided by the manufacturer which employs cumulants analysis and non-negatively constrained least-squares particle size distribution analysis was used to determine the intensity-mean z-averaged particle diameter (D_{eff}), polydispersity index (PDI), and ζ -potential. All measurements were performed at least in triplicate to calculate mean values $\pm$ SD. Complexes were prepared in various buffer systems of 10mM concentration: citrate buffer - pH 5 and 6; phosphate buffer - pH 7, HEPES – pH 8, and Tris buffer - pH 9. Concentration of peptide in the complexes was 50 μ M.

2.4 Atomic Force Microscopy (AFM) Analysis

Samples for AFM imaging were prepared by depositing 5 μ l of an aqueous dispersion of complexes (0.125 mg/ml) onto positively charged 1-(3-aminopropyl) silatrane mica surface (APS-mica) or freshly cleaved negatively charged mica for 2 minutes followed by surface washing with deionized water and drying under argon atmosphere. The AFM imaging in air was performed with regular etched silicon probes with a spring constant of 42 N/m using a Multimode NanoScope IV system (Veeco, Santa Barbara, CA) operated in a tapping mode. The images were processed, and the widths and heights of the particles were measured using Femtoscan software (Advanced Technologies Center, Moscow, Russia).

2.5 Circular dichroism (CD) spectroscopy

The CD spectra were recorded by using an Aviv circular dichroism spectrometer (model 202SF, Aviv Associates Inc., Lakewood, NJ). The spectra were measured at 25 °C using a 1 mm pathlength cell over a wavelength range from 190 to 300 nm in 10 mM phosphate buffer (pH 7.0) with and without 0.14 M NaCl. Data were collected at 1 nm intervals with a scan rate of 15 nm/min. All spectra were acquired in triplicate and averaged. The spectrum of an appropriate buffer control sample was then subtracted from each of the sample spectra. The final spectral data were converted to mean molar ellipticities. The peptide concentrations were 100 μ M.

2.6 Trypsin digestion analysis

APN stability against proteolytic digestion was studied using Trypsin Spin Columns (Sigma). Columns were first prepared by washing with enzyme reaction buffer and

centrifugation according to the product protocol. For sample digestion, 100 μ l of buffered solution of p41, corresponding APN ($Z_{-/+}=2$), or PEG-PLD₂₀ (20 μ g based on peptide equivalents) were applied on the trypsin spin columns. pH was kept at approximately 8 during the whole procedure. The samples in the column were incubated at room temperature for two time points, 5 min and 13 min, respectively, followed by elution with enzyme reaction buffer or water and centrifugation at 3000 rpm for 2 min. Eluates were analyzed by the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and gel electrophoresis. The MALDI-TOF MS measurement was performed by UNMC mass spectrometry and proteomics Core Facility.

2.7 Hemolytic activity

Human blood was collected in BD heparinized vacutainers, centrifuged at 13500 $\times$ g to separate the red blood cells (RBC). The pellet was washed three times using 150 mM saline solution. After the third wash, the RBC solution was diluted with 100 mM PBS solutions to make the final RBC concentration into 10⁸ per 200 μ l while maintaining pH at 7.4. APN, peptides, or copolymer were added to 200 μ l of RBC suspensions at different concentrations (2.5 μ M, 5 μ M and 10 μ M on peptide basis), gently mixed and incubated for 60 min in a 37°C water bath. The release of hemoglobin was determined after centrifugation (13,500 $\times$ g for 5 min) by spectrophotometric analysis of the supernatant at 541 nm. The hemolysis of RBC in PBS solutions and in 1% v/v Triton X-100 solution were used as negative and positive controls, respectively. The observed hemolytic activity of each tested compound was normalized to that of the positive control, 1% v/v Triton X-100 solution, as a 100% hemolysis.

2.9 Immunofluorescent staining and confocal microscopy

Monocytes were grown in suspension cultures using Teflon flasks or cultured as adherent cells in poly-D-lysine/fibronectin-coated LabTek chamber slides were purchased from BD Biosciences (San Diego, CA). Rabbit Abs to EEA1 were purchased from Cell Signaling Technologies (Danvers, MA). Rabbit monoclonal Abs against PEG were purchased from Epitomics, Inc. (Burlingame, CA). The secondary Abs conjugated to Alexa Fluor 488, 594 and ProLong Gold anti-fading solution with 4',6-diamidino-2-phenylindole (DAPI) were obtained from Life Technologies Corporation (Grand Island, NY). For immunofluorescent staining, cells were washed three times with PBS and adherent cells were fixed with 4% paraformaldehyde solution (PFA) in PBS at room temperature for 30 min. Cells were treated with blocking/permeabilizing solution (0.1% Triton, 5% BSA in PBS) and quenched with 50 mM NH₄Cl for 15 min. Cells were washed once with 0.1% Triton in PBS and sequentially incubated with primary and secondary Abs at room temperature. Non-specific cross binding of secondary Abs was tested prior to immunostaining. Slides were covered in ProLong Gold anti-fading reagent with DAPI and imaged using a 63X oil lens in a LSM 510 confocal microscope (Zeiss). Cell suspension was stained for FACS utilizing BD Bioscience protocol for intracellular staining (cat. # 560098) and samples were analyzed by BD FACSArray™ Bioanalyzer.

2.10 In vitro anti-HCV activity

The infectious full-length genotype 2a HCV clone JFH1 that replicates and produces infectious virus particles in cell culture was used in these studies as previously described [28]. Huh-7.5 cell line provided by Dr. Charles Rice (The Rockefeller University, New York, NY) was propagated in DMEM supplemented with 10% fetal bovine serum and 1% nonessential amino acids. Cells were infected with JFH1 virus (MOI 0.1) overnight, virus was removed and cells were incubated for 7 days in fresh media. The peptides (10 mM stock solution in DMSO), copolymer and APN were diluted to peptide equivalent concentrations of 10, 5 and 2.5 μ M, incubated in complete culture medium with cells for 1 h and then were

removed before infection (single pretreatment) or were added to the culture medium post infection every 48 h with media exchange (multiple treatments). To confirm the intracellular levels of HCV infection, real-time PCR was performed as described [29]. In brief, HCV RNA quantification was performed using a StepOne Realtime PCR system amplifying a highly conserved sequence in the 5' UTR of the viral genome. Total viral RNA was extracted using the MagMax Viral RNA Isolation Kit (Applied Biosystems), and first-strand cDNA synthesis performed using a high capacity RNA-to-cDNA kit (Applied Biosystems). The following primers and probe for this consensus sequence were designed using PrimerExpress Software v2.0 (Applied Biosystems): 5'UTRF GACCGGGTCCTTCTTGGAT; 5'UTRR CCAACACTACTCGGCTAGCAGTCT; probe FAM-ATTTGGGCGTGCCCCGCNFQ. Positive and negative controls were included in all runs. To measure the intracellular expression of HCV core protein by flow cytometry infected Huh7.5 cells were detached by EDTA-containing Cell stripper and then permeabilized using BD Pharmingen buffer set (cat # 560098). After permeabilization, cells were stained with monoclonal antibody to HCV core protein 1 μ l/well (clone C7-50, Thermo Fisher Scientific Inc., IL, USA). After 1 hr incubation, cells were additionally stained with anti-mouse IgG-PE and analyzed by FACSDiva (BD Biosciences Immunocytometry Systems).

2.11 In vitro anti-HIV-1 activity

Human monocytes and peripheral blood lymphocytes (PBL) were obtained from leukopheresis of HIV-1, HIV-2 and hepatitis B seronegative donors and purified by countercurrent centrifugal elutriation as previously described [30]. Monocyte-derived macrophages (MDM) were cultured in DMEM supplemented with 10% heat-inactivated pooled human serum and 1% glutamine (Sigma-Aldrich, St. Louis, MO), 10 mg/ml ciprofloxacin (Sigma-Aldrich), and 1000 U/ml of purified recombinant human macrophage colony stimulating factor (M-CSF) [30]. The CCR5 coreceptor utilizing HIV-1_{ADA} strain was propagated using MDM. CXC4-utilizing lymphocytotropic HIV-1_{LAI} strain was propagated on phytohemagglutinin (PHA)-stimulated PBL in the presence of interleukin-2 (BD Bioscience, San Jose, CA) (PHA/IL-2 lymphoblasts). Cells were split 1:2 and infected 3 days after stimulation. Viral preparations were screened and found to be negative for endotoxin (<10 pg/ml) (Associates of Cape Cod, Woods Hole, MA) and mycoplasma (Gen-Probe II; Gen-Probe, San Diego, CA). PBL and MDM cell cultures were infected with HIV-1 at MOI 0.01.

To test drug efficacy TZM-bl cells (JC53BL-13, NIH AIDS Research and Reference Reagent Program) were cultured in DMEM supplemented with 10% fetal bovine serum, 100 units of penicillin and 100 μ g/ml of streptomycin. Cells were allowed to reach 70% confluence, then detached by 25 mM trypsin/EDTA for 5 min at 37 °C. Detached cells were seeded in 96-well plates at 1×10^4 cell/well and used in experiments when they reached 40% confluence. Cells were pretreated with drugs (copolymer, p1, p41 and APN) at peptide equivalent concentrations of 2.5, 5 and 10 μ M for 2 h, washed and infected. After infection cells were cultured for additional 48 h prior to β -galactosidase-positive cell number detection (per manufacturer's instructions, Invitrogen). Bright field images were acquired using a Nikon Eclipse TE300 microscope (Nikon) and virus-infected blue cells were counted. PHA/IL-2 lymphoblasts were pretreated with APN and peptides at a concentration of 2.5 and 5 μ M for 2 h and infected with HIV-1_{LAI} for 4 h, then drugs and inoculum were removed and media with drugs were added for 4 consecutive days. All treatments were done in quadruplicates. MDM were plated in 96-well flat-bottom plates (10^6 cells/ml), matured in the presence of M-CSF for 7 days and used in 6 parallels for all treatments. Cells were incubated with peptide, copolymer and APN at 10 μ M for 2 h and drugs were removed before infection, or were added to the culture medium every 48 h with media exchange.

Supernatants were collected at days 5, 7 and 10 post infection. HIV-1 replication in MDM and PBL cell cultures was detected by reverse transcriptase (RT) activity [31] and adjusted to the cell viability determined by the standard tetrazolium dye method (MTT assay).

2.12 In vivo anti-HIV-1 activity

In vivo anti-HIV-1 activity was tested on 4-week-old NOD/*scid*- γ_c^{null} mice (NSG, The Jackson Laboratories, stock # 005557) reconstituted with human peripheral blood lymphocytes (hu-PBL-NSG) as described before [32–34]. Briefly, hu-PBL 20×10^6 cells/mouse were injected intraperitoneally (i.p.), 7 days later animals were injected in the caudal thigh muscle intramuscularly (i.m.) with p41 or APN (at a dose of 50 μ g of peptide in 50 μ l volume). Control animals received saline. One group of mice was left uninfected, three other groups (treated with saline, p41 or APN, $n = 6$ per group) were inoculated i.p. with HIV-1_{ADA} at 10^4 of 50% tissue culture infectious doses (TCID₅₀) 30 minutes later. APN, p41 or saline were administered for the next 6 days i.m. and animals were sacrificed 24 h after the last i.m. injection. Spleen tissue samples were collected for flow cytometry analysis and RNA extraction. *HIV-1 gag* RNA expression was determined using real time PCR assays with primers and probes previously described [35]. All PCR reagents were obtained from Applied Biosystems. Gene expression was normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which was used as an endogenous control.

2.13 Flow Cytometry

APN and p41 uptake by macrophages was determined by FACS. In a 96-round-bottom plate MDM and compounds were incubated at 37°C for 5 - 120 min. Plate was transferred on ice, cells were washed with ice-cold buffer, and intracellular staining for PEG was performed for cells treated with APN and PEG-PLD₂₀. Cells exposed to p41-Cy5 were washed and fixed in 2% PFA solution.

FACS analysis of animal splenocytes was conducted as previously outlined by Gorantla and colleagues [36]. In short, spleens were extracted from the mice at sacrifice and crushed through a 40- μ m cell strainer to obtain single cell suspensions. Splenocytes thus isolated were stained for human cells using antibodies to CD45, CD3, CD4, CD8. Appropriate isotype controls were used, and all antibodies were obtained from BD Pharmingen (San Diego, CA, USA). Cells were analyzed using BD LSR II with BD FACS Diva software (BD Immunocytometry Systems, Mountain View, CA, USA). All animals had comparable levels of PBL engraftment.

2.14 In vivo evaluation of toxicity and immunogenicity of peptides and APN

Toxicity and immunogenicity of peptide and APN were tested on C57Bl/6 mice by 7 daily i.m. administrations (50 μ g peptide or equivalent dose of APN, $n = 3$ per group) with two week follow up observation. Serum was collected for the detection of antibodies to peptide/polymer by ELISA. ELISA plates were coated with 100 μ g/ml of p41, APN and polymer in phosphate buffer solution overnight, washed and blocked with 3% bovine serum albumin for 1 h. Serial dilutions (1:20 – 1:2400) of heat-inactivated serum were added for 2 h. Anti-mouse IgM and anti-mouse IgG were detected with reagents and protocol obtained from Bethyl Laboratories, Inc. (Montgomery, TX). Reaction was calculated as differences in end point titers between experimental and saline-treated animals. Tissues (liver, kidney, lung, spleens and brains) were collected in 4% PFA for fixation, embedded in paraffin and analyzed after H&E staining for pathomorphological changes.

2.15 Statistical analysis

Data were analyzed using ANOVA and Student's *t* test for comparisons. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1 Characteristics of APN

P41 peptide has a net charge of +6 at pH 7 and is therefore cationic at physiological conditions. Peptide - block ionomer complexes (APN) were prepared by simple mixing of buffered solutions (10 mM phosphate buffer, pH 7 or PBS pH 7.4) of p41 and anionic block copolymer (PEG-PLD or PEG-PLE), which electrostatically bind to each other (Fig. 1A). Formation of complexes was confirmed by gel filtration chromatography, sedimentation equilibrium analysis (Supplementary information, Fig. S1 and Fig. S2), and DLS (Table 2). P41 was almost completely incorporated into the complexes at the stoichiometric composition of the mixtures. The APN particles were found to be very small (average diameter of approximately 35 nm), uniform (monomodal, relatively narrow particle size distribution with polydispersity indices (PDI) in the range of 0.1 – 0.2), and had slightly negative ζ -potential. In a sharp contrast, p41 had a tendency to form positively charged large aggregates (around 900 nm in diameter) in diluted aqueous solutions. The complexes formed by the block ionomer with longer ionic chains (PEG-PLE₄₀) appear to be larger. Furthermore, the systems formed by this block ionomer were more polydisperse. The formation of nanosized APN was further confirmed by AFM. A typical image of p41/PEG-PLD₂₀ APN is shown in Fig. 1B. Analysis of the images revealed that APN appeared to be round-shaped particles with a narrow distribution in size and an average of height of approximately 2–3 nm and width in the range of 20 - 40 nm depending on copolymer structure. It should be noted that imaging in air usually provides lower numbers for the height because of the drying process, but provides higher numbers for the width, due to the tip-convolution effect. Furthermore, interaction between particles and positively charged mica surface might also result in additional flattening and affect dimensions measured by AFM. In contrast to APN, free p41 peptide deposited on negatively charged mica form large non-structured aggregates (Fig. 1B). These observations were in good agreement with the DLS data.

The dimensions of APN particles in aqueous dispersions practically did not change upon pH variation between pH 5–9 (Supplementary information, Table S1). APN maintained their colloidal stability and exhibited no aggregation for a prolonged period of time (at least two weeks) in the absence of salt (10 mM phosphate buffer) and within 24 h at physiological concentrations of salt (0.14 M NaCl). Beyond this time formation of heterogeneous particle population was detected by DLS followed by slow aggregation (Table S2).

As expected, CD spectra of the p41 in aqueous solution indicated a notable degree of helical structure (Fig. 2 and Fig. S3). It exhibited a double minima at ~208 and 222 nm along with a positive ellipticity at ~195 nm, features that are typical of α -helices. The absolute magnitude of these peaks was markedly increased upon binding of p41 to PEG-PLD₂₀. Importantly, PEG-PLD₂₀ copolymer at these conditions is characterized by a single broad negative ellipticity centered at ~202 nm, indicative of an unordered structure. These data suggest that incorporation of p41 into APN did not only alter the inherent propensity of the peptides to form α -helical structures but also enhanced the helical content of p41, which is necessary for their antiviral activity.

3.2 Stability against trypsin digestion

APN stability against proteolytic digestion was studied using Trypsin Spin Columns followed by MALDI-TOF MS (Fig. 3). The incorporation of p41 into APN resulted in partial protection of peptide against proteolytic digestion by trypsin. Indeed, the MS spectra of p41/PEG-PLD₂₀ APN digested for 5 and 13 min showed the existence of intact p41 with the peak at 2433 (m/z) along with the peaks belong to the p41 fragments (identified as SWLR and IWR, respectively). In contrast, at similar conditions p41 alone was completely disintegrated. Higher stability of APN against protease degradation was additionally confirmed by gel electrophoresis (Fig. S4).

3.3 Hemolytic activity of APN

Considering that cationic p41 is toxic to negatively charged cell membranes, the red blood cell hemolysis assay of p41 and APN was conducted to evaluate their membrane destabilizing activity (Fig. 4). In this study 1% v/v Triton X-100 and PBS were adopted as the control groups for 100% and 0% hemolysis, respectively. Anionic block copolymers and parental p1 peptide (a net charge of -2 at pH 7) did not cause significant levels of hemolysis at all concentrations in the experimental range. As expected, p41 induced hemolysis in a dose-dependent manner. At 10 μ M concentrations p41 revealed $56.70 \pm 0.03\%$ of hemolysis. Regardless of concentration, the hemolytic activity of p41 immobilized in APN was significantly reduced compared to free p41.

3.4 Cellular uptake of APN

Cellular uptake of APN was evaluated in Huh7.5 hepatoma cells and monocyte-derived macrophages (MDM). Exposure of Huh7.5 cells to Cy5-labeled p41 led to significant intracellular accumulation of free peptide. However, after continuous incubation for 4 h the damage of cell monolayer was observed and cells started to undergo apoptotic death (Fig. 5) suggesting the cytotoxic activity of the free cationic peptide. In contrast, APN were less efficiently taken by Huh7.5 cells, but did not induce cytotoxicity or affect the monolayer integrity (Fig. 5). The accumulation kinetics in MDM, highly active phagocytic cells, suggested a rapid and time-dependent uptake of APN while uptake of PEG-PLD₂₀ copolymer alone was negligible (Fig. 6A). Notably the free peptide was taken up by MDM almost completely in 5 min (95% of MDM were Cy5-positive). The reduced uptake efficiency of APN at the same time point (45.8% of MDM were loaded with APN) can be attributed to the steric hindrance of PEG chains on the surface of the particles that prevent their interactions with the cells. In order to understand the mechanisms of APN uptake we utilized the attached culture of MDM (Fig. 6 B, C). Cells were co-incubated with APN for 5 - 120 min, washed, fixed and stained with antibodies to PEG and early endosome antigen 1 (EEA1). PEG-positive granules were colocalized with the surface cellular membranes by 5 min of incubation (data not shown). After 2 h PEG-positive staining of surface membranes increased and intracellular colocalization with EEA1 was found. This suggests that APN could be endocytosed by early endosomes and possibly sorted to late endosomes and lysosomes, as well as for recycling to the plasma membrane.

3.5 Anti-HCV activity of peptides and APN

Anti-HCV activity of p41 and APN was evaluated against JFH1 virus (MOI 0.1) in Huh7.5 cell line. The cell infectivity and antiviral potency of formulations were determined using RT-PCR (HCV-RNA expression) and FACS analysis (staining of intracellular HCV core protein). As shown in Fig. 7A, a significant reduction in intracellular viral RNA was detected in Huh7.5 cells pretreated for 1 h with APN (p41/PEG-PLD₂₀, at a dose equivalent to 10 μ M p41) prior to HCV infection compared to infected control cells. Notably, the potency of p41 to inhibit the establishment of HCV infection was significantly enhanced

after its incorporation into APN compared to free peptide. To assess whether APN can suppress an ongoing HCV infection, the cells were incubated for 1 h with different doses of APN and then infected with virus and cultured in the presence of APN for 7 days (APN at the corresponding concentrations were replenished at culture medium exchange every 48 h). As shown in Fig. 7B, the APN decreased the number of HCV infected cells in concentration-dependent manner with no sign of toxicity, and at a dose equivalent to 10 μ M p41 the infected cells were completely eradicated. It is worth mentioning that prolonged exposure of the infected cells to free peptides in similar treatment regimen resulted in extensive cell loss, again confirming the propensity of APN to suppress the cytotoxicity of cationic peptide. Overall, these data suggest that APN are able to inhibit HCV infection both extracellularly and within infected cells.

3.7 Anti-HIV activity of peptides and APN in vitro

As was shown HCV C5A protein-derived peptide (in our studies designated as p1) added with HIV-1 to TZM-bl cells for 4 h prevents HIV infection via neutralization (destabilization) of both free and cell-bound viral particles [20, 21]. We tested the ability of the peptides (both p1 and p41), copolymer alone, and APN (p41/PEG-PLD₂₀) to prevent infection after 2 h pretreatment of cells, assuming prolonged peptide activity in the complex. We found that pretreatment of TZM-bl cells with copolymer alone or p1 did not prevent the infection. Interestingly, in this cell model p41 exhibited stronger antiviral activity compared to parental p1 peptide that was further significantly enhanced by its incorporation in APN (Fig. 8A). Also, pretreatment of activated PBL for 2 h before infection following by incubation of cells with 2.5 and 5 μ M of APN (on p41 basis) had superior activity compared to the free peptides (Fig. 8B). When MDM were pretreated for 2 h with 10 μ M of peptides or APN before infection and drugs were added every other day with culture medium exchange, only APN suppressed viral replication by more than 90% (Fig. 8C). It is also important to note that similarly to Huh7.5 cells the multiple treatments of infected MDM with free peptide were toxic to the cells while no significant changes in MDM viability were detected upon treatments with APN (Fig. S5). These data suggest that incorporation of p41 into APN substantially potentiate and prolongate an antiviral activity of peptide against HIV.

3.8 In vivo anti-HIV activity of APN

Initially, the toxicity of APN and their constituents was assessed by i.m. administration in 6-week-old C57Bl/6 mice. Animals were inoculated with the corresponding formulation everyday over the 7 days followed by two-week observation of their well-being. No alterations in animal behavior, body weight or hypersensitivity reactions were observed during the experiment. Histopathological analysis by light microscopic examination of H&E stained tissue sections did not reveal any pathological changes as a result of treatments.

As a proof of concept that APN can suppress HIV-1 replication after systemic administration *in vivo*, we performed an experiment where NSG mice were transplanted with human hu-PBL. In 30 min prior to i.p. virus inoculation mice were injected i.m. with 50 μ g of free p41 or its APN format. Animals were then treated with p41 or APN on daily basis for the next six days, and were euthanized on day 7 post infection. An additional group of animals was treated with saline as a control. As shown in Fig. 9, within 7 days the total number of human lymphocytes in the spleen of animals infected with HIV-1 was not significantly reduced compared to uninfected animals (Fig. 9A). However, the number of CD3+CD4⁺ cells significantly declined compared to uninfected control group ($24.8 \pm 0.6\%$ versus $32.6 \pm 2.5\%$, $p=0.033$) (Fig. 9B, C). The drop in CD4:CD8 cell ratio was also observed in infected animals (0.38 ± 0.01 versus 0.56 ± 0.09 for uninfected group, $p=0.013$) suggesting the loss of CD4⁺ cells due to HIV infection (Fig. 9D). Consistent with these results, the presence of high levels of *HIVgag* RNA expression was detected within

spleen tissue of the infected animals (Fig. 9E). These readouts, known to be associated with HIV-1 infection in hu-PBL-NSG mice, were all affected by treatments with APN. Significant reduction in viral RNA expression was detected in spleen tissue of animals treated with either APN or p41 alone (Fig. 9E). However, APN treatment was more effective in inhibition of virus replication. Indeed, the median normalized to GAPDH RT-PCR values in APN-treated group was 2.08 ($p = 0.004$) compared to 690 in HIV-infected control group, while the p41 treatment reduced this value only to 120 ($p = 0.026$). In particular, in four of six animals in APN-treated group the *HIVgag* expression was significantly suppressed $\sim$ by 2 log₁₀ (in the range of 0.63 to 2.48). In agreement with APN anti-viral effects the percentage of CD3+CD4+ cells in this group was not different from uninfected and significantly higher than in infected or p41-treated animals (Fig. 9B, C). Although treatment with p41 alone resulted in reduced viral RNA expression, it was not protective for CD3+CD4+ cell number. A similar pattern was observed for CD4:CD8 cell ratio (Fig. 9D). Thus, these observations confirm that APN possess anti-HIV activity *in vivo*.

4. Discussion

A virocidal peptide (C5A) was found to be effective in inhibition of HCV and HIV infections as well as other *Flaviviridae* members *in vitro* [21]. This peptide derived from the membrane anchor domain of the HCV nonstructural protein NS5A prevents an initiation of HCV infection by destroying the virus and suppresses ongoing infections by blocking the cell-to-cell spread of the virus. It was suggested that C5A recognizes cellular components of virus membranes, most likely their lipid composition. A cationic derivative of this peptide (p41) was also found to display virocidal activity against HCV. The limitations to the use of these peptides as therapeutics are their rapid elimination from circulation, inactivation by proteases present in the body, as well as unfavorable toxicity profile typical for cationic peptides. To overcome these restraints and ensure prolonged stability of p41 molecule in an active form, the cationic antiviral peptide p41 was incorporated into polyion complexes, APN, with anionic biodegradable PEG-poly(amino acid) block copolymers (Fig. 1A). Electrostatic coupling of the negatively charged carboxylic groups of the block copolymer and positively charged amino groups of p41 leads to the formation of hydrophobic domains, which segregate in aqueous media into a peptide/polyion core of polyion complex micelles. Water-soluble nonionic segments (here PEG) prevent aggregation and macroscopic phase separation. As a result, these APN self-assemble into particles of nanoscale size and form stable aqueous dispersions at the physiological conditions (pH, ionic strength). We found that the length of the anionic segment of the block copolymer need to be around 10–20 monomer units to ensure the formation of well-defined particles with unimodal distribution and low polydispersity. At these conditions the sizes, charge and morphology of the resulting APN did not depend on the chemical structure of the block copolymers. This study demonstrated that binding of p41 to the block ionomer resulted in stabilization of α -helical structure of the peptide that is functionally linked to its virocidal activity. Notably, incorporation of p41 into the cores of the complexes was able to significantly reduce cytotoxicity and hemolytic activity associated with cationic peptides. Moreover it delayed proteolytic degradation of the peptide. The enhanced stability of APN against inactivation by proteases in combination with decreased cytotoxicity may result in extended circulation time and allow their administration at higher doses.

APN demonstrated time-dependent cellular accumulation in both macrophage and Huh7.5 cell models. PEGylation is known to reduce interaction of particles with cells due to the formation of a hydrophilic stealth coating around the particles leading to reduced uptake [37]. Thus, it was not surprising that APN were taken up by the cells at a slower rate than free peptide. Our data suggest that following the entry in macrophages APN trafficked to early endosomes. Both viruses are known to exploit cell-encoded pathways of intercellular

vesicle trafficking, exosome exchange, for both the biogenesis of viral particles and transmission [38–43]. Thus, it is likely that APN might pursue intracellular pathways similar to viruses and destroy them in earlier endosomes or other endocytic compartments before they enter the cytoplasm or are degraded in lysosomes. Similarly as non-formulated peptide, APN were able to inactivate both HIV and HCV upon direct interaction in media. The infectivity of the viruses treated in such a way was significantly reduced. We also observed that APN retained intracellular anti-HCV and anti-HIV activity. Notably, APN significantly exceeded antiviral activity of the non-modified peptides when cells were infected after pretreatment with APN. The prolonged antiviral effect of APN in cells (up to 48 h post-withdrawal of the treatment) is most likely due to its improved stability. We believe that the block copolymer chains in the APN can sterically protect p41 molecules against degradation by intracellular proteases. This is further supported by the fact that in spite of the lower uptake of APN compared to free peptide, the internalized fraction remained more active over time. When administered *in vivo* APN appeared to be well tolerated by the animals, as judged by their general behavior and absence of signs of toxicity. APN was able to suppress viral replication and prevent loss of CD4⁺ cells in spleen of the treated mice, while naked peptide did not have such CD4⁺ cell protective activity. Overall, we demonstrated that the antiviral peptides incorporated into the protective polymer scaffold exhibited increased stability, bioavailability, retained virocidal activity, and have therapeutic potential. Despite the absence of histological evidences of toxicity, the observed high levels of p41 in *vitro* hemolytic activity and possibility to induce the death of activated human lymphocytes, cannot be excluded and may preclude the therapeutic application of cationic p41 peptide even in form of APN. Nevertheless, presented data supports the hypothesis that incorporation of antiviral peptides into block ionomer complexes can address the challenges of protein therapeutic delivery by improving stability, reducing toxicity, and increasing bioavailability.

Conclusions

Well-defined nanocomplexes of positively charged antiviral amphipathic α -helical peptides were prepared by electrostatic coupling with anionic biodegradable block copolymers based on poly(amino acids). Our *in vitro* studies suggest that incorporation of cationic peptides into APN substantially attenuate their intrinsic cytotoxicity while preserving an antiviral activity against both HIV and HCV viruses. As a proof-of-concept, we demonstrated that APN were able to decrease the viral load in mice transplanted with human lymphocytes and HIV-1-infected without signs of toxicity to the animals. The unique self-assembly behavior and the simplicity of the preparation make the APN approach an extremely promising platform for the delivery of therapeutic peptides. The distinctive virocidal mechanisms of action of amphiphatic α -helical peptide and proposed strategy of therapeutic delivery by APN may provide a potentially broad applicability in combination with standard therapeutics for HIV/HCV in cases of viruses drug resistance, advanced end-stage liver disease, and for the reduction of HCV viral rebound after liver transplantation for monoinfected patients. APN inclusion in therapeutic strategies may also shorten the treatment of HCV monoinfected patients in IFN- α non-responders and IFN- α -free regimens.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was supported by National Institutes of Health (NIH) COBRE grant RR021937 (Nebraska Center for Nanomedicine) to L.Y.P. and T.K.B., and CA116590 to T.K.B. The authors are grateful to Dr. Irine Khutsishvili for her assistance with CD spectroscopy, Dr. Samuel Sanderson for the help with peptide purification and Dr. Santhi Gorantla for PCR analysis. We acknowledge the assistance of the COBRE Nanomaterials Core facility and UNMC Nanoimaging Core facility.

References

1. Deming P, McNicholl IR. Coinfection with human immunodeficiency virus and hepatitis C virus: challenges and therapeutic advances. *Insights from the Society of Infectious Diseases Pharmacists. Pharmacotherapy.* 2011; 31:357–368. [PubMed: 21449625]
2. Alter MJ. Epidemiology of viral hepatitis and HIV co-infection. *J Hepatol.* 2006; 44:S6–S9. [PubMed: 16352363]
3. Staples CT Jr, Rimland D, Dudas D. Hepatitis C in the HIV (human immunodeficiency virus) Atlanta, V.A. (Veterans Affairs Medical Center) Cohort Study (HAVACS): the effect of coinfection on survival. *Clin Infect Dis.* 1999; 29:150–154. [PubMed: 10433578]
4. Denissen JF, Grabowski BA, Johnson MK, Buko AM, Kempf DJ, Thomas SB, et al. Metabolism and disposition of the HIV-1 protease inhibitor ritonavir (ABT-538) in rats, dogs, and humans. *Drug Metab Dispos.* 1997; 25:489–501. [PubMed: 9107549]
5. Puoti M, Manno D, Nasta P, Carosi G. The burden of HIV and hepatitis C virus coinfection. *Curr Opin HIV AIDS.* 2007; 2:460–465. [PubMed: 19372928]
6. Chen TY, Ding EL, Seage Iii GR, Kim AY. Meta-analysis: increased mortality associated with hepatitis C in HIV-infected persons is unrelated to HIV disease progression. *Clin Infect Dis.* 2009; 49:1605–1615. [PubMed: 19842982]
7. Buskin SE, Barash EA, Scott JD, Aboulafia DM, Wood RW. Hepatitis B and C infection and liver disease trends among human immunodeficiency virus-infected individuals. *World J Gastroenterol.* 2011; 17:1807–1816. [PubMed: 21528052]
8. Rosenthal E, Poiree M, Pradier C, Perronne C, Salmon-Ceron D, Geffray L, et al. Mortality due to hepatitis C-related liver disease in HIV-infected patients in France (Mortavic 2001 study). *AIDS.* 2003; 17:1803–1809. [PubMed: 12891066]
9. Lai AR, Tashima KT, Taylor LE. Antiretroviral medication considerations for individuals coinfecting with HIV and hepatitis C virus. *AIDS Patient Care STDS.* 2006; 20:678–692. [PubMed: 17052138]
10. Daar ES, Lynn H, Donfield S, Gomperts E, O'Brien SJ, Hilgartner MW, et al. Hepatitis C virus load is associated with human immunodeficiency virus type 1 disease progression in hemophiliacs. *J Infect Dis.* 2001; 183:589–595. [PubMed: 11170984]
11. Ragni MV, Nalesnik MA, Schillo R, Dang Q. Highly active antiretroviral therapy improves ESLD-free survival in HIV-HCV co-infection. *Haemophilia.* 2009; 15:552–558. [PubMed: 19347994]
12. Reiche EM, Bonametti AM, Morimoto HK, Morimoto AA, Wiechemann SL, Matsuo T, et al. Epidemiological, immunological and virological characteristics, and disease progression of HIV-1/HCV-co-infected patients from a southern Brazilian population. *Int J Mol Med.* 2008; 21:387–395. [PubMed: 18288387]
13. Seden K, Back D, Khoo S. New directly acting antivirals for hepatitis C: potential for interaction with antiretrovirals. *J Antimicrob Chemother.* 2010; 65:1079–1085. [PubMed: 20335191]
14. Shivraj SO, Chattopadhy D, Grover G, Kumar A, Baveja UK. Role of HCV coinfection towards disease progression and survival in HIV-1 infected children: a follow-up study of 10 years. *J Trop Pediatr.* 2006; 52:206–211. [PubMed: 16339160]
15. Serpaggi J, Chaix ML, Batisse D, Dupont C, Vallet-Pichard A, Fontaine H, et al. Sexually transmitted acute infection with a clustered genotype 4 hepatitis C virus in HIV-1-infected men and inefficacy of early antiviral therapy. *AIDS.* 2006; 20:233–240. [PubMed: 16511416]
16. Vallet-Pichard A, Pol S. Natural history and predictors of severity of chronic hepatitis C virus (HCV) and human immunodeficiency virus (HIV) co-infection. *J Hepatol.* 2006; 44:S28–S34. [PubMed: 16343684]

17. Rallon NI, Naggie S, Benito JM, Medrano J, Restrepo C, Goldstein D, et al. Association of a single nucleotide polymorphism near the interleukin-28B gene with response to hepatitis C therapy in HIV/hepatitis C virus-coinfected patients. *AIDS*. 2010; 24:F23–F29. [PubMed: 20389235]
18. Alestig E, Arnholm B, Eilard A, Lagging M, Nilsson S, Norkrans G, et al. Core mutations, IL28B polymorphisms and response to peginterferon/ribavirin treatment in Swedish patients with hepatitis C virus genotype 1 infection. *BMC Infect Dis*. 2011; 11:124. [PubMed: 21569441]
19. Graham CS, Wells A, Liu T, Sherman KE, Peters M, Chung RT, et al. Relationships between cellular immune responses and treatment outcomes with interferon and ribavirin in HIV/hepatitis C virus co-infection. *AIDS*. 2006; 20:345–351. [PubMed: 16439867]
20. Bobardt MD, Cheng G, de Witte L, Selvarajah S, Chatterji U, Sanders-Beer BE, et al. Hepatitis C virus NS5A anchor peptide disrupts human immunodeficiency virus. *Proc Natl Acad Sci USA*. 2008; 105:5525–5530. [PubMed: 18378908]
21. Cheng G, Montero A, Gastaminza P, Whitten-Bauer C, Wieland SF, Isogawa M, et al. A virocidal amphipathic {alpha}-helical peptide that inhibits hepatitis C virus infection in vitro. *Proc Natl Acad Sci U S A*. 2008; 105:3088–3093. [PubMed: 18287023]
22. Lin Q, Fang D, Hou X, Le Y, Fang J, Wen F, et al. HCV peptide (C5A), an amphipathic alpha-helical peptide of hepatitis virus C, is an activator of N-formyl peptide receptor in human phagocytes. *J Immunol*. 2011; 186:2087–2094. [PubMed: 21228351]
23. Batrakova EV, Li S, Reynolds AD, Mosley RL, Bronich TK, Kabanov AV, et al. A macrophage-nanozyme delivery system for Parkinson's disease. *Bioconjug Chem*. 2007; 18:1498–1506. [PubMed: 17760417]
24. Gaydoss A, Duysen E, Li Y, Gilman V, Kabanov A, Lockridge O, et al. Visualization of exogenous delivery of nanoformulated butyrylcholinesterase to the central nervous system. *Chem Biol Interact*. 2010; 187:295–298. [PubMed: 20060815]
25. Harada A, Kataoka K. Pronounced activity of enzymes through the incorporation into the core of polyion complex micelles made from charged block copolymers. *J Control Release*. 2001; 72:85–91. [PubMed: 11389987]
26. Yuan X, Harada A, Yamasaki Y, Kataoka K. Stabilization of lysozyme-incorporated polyion complex micelles by the omega-end derivatization of poly(ethylene glycol)-poly(alpha,beta-aspartic acid) block copolymers with hydrophobic groups. *Langmuir*. 2005; 21:2668–2674. [PubMed: 15779933]
27. Lee Y, Fukushima S, Bae Y, Hiki S, Ishii T, Kataoka K. A protein nanocarrier from charge-conversion polymer in response to endosomal pH. *J Am Chem Soc*. 2007; 129:5362–5363. [PubMed: 17408272]
28. Lindenbach BD, Evans MJ, Syder AJ, Wolk B, Tellinghuisen TL, Liu CC, et al. Complete replication of hepatitis C virus in cell culture. *Science*. 2005; 309:623–626. [PubMed: 15947137]
29. Mercer DF, Schiller DE, Elliott JF, Douglas DN, Hao C, Rinfret A, et al. Hepatitis C virus replication in mice with chimeric human livers. *Nat Med*. 2001; 7:927–933. [PubMed: 11479625]
30. Gendelman HE, Orenstein JM, Martin MA, Ferrua C, Mitra R, Phipps T, et al. Efficient isolation and propagation of human immunodeficiency virus on recombinant colony-stimulating factor 1-treated monocytes. *J Exp Med*. 1988; 167:1428–1441. [PubMed: 3258626]
31. Reardon JE, Miller W. Human immunodeficiency virus reverse transcriptase. Substrate and inhibitor kinetics with thymidine 5'-triphosphate and 3'-azido-3'-deoxythymidine 5'-triphosphate. *J Biol Chem*. 1990; 265:20302–20307. [PubMed: 1700787]
32. Poluektova LY, Munn DH, Persidsky Y, Gendelman HE. Generation of cytotoxic T cells against virus-infected human brain macrophages in a murine model of HIV-1 encephalitis. *J Immunol*. 2002; 168:3941–3949. [PubMed: 11937550]
33. Gorantla S, Santos K, Meyer V, Dewhurst S, Bowers WJ, Federoff HJ, et al. Human dendritic cells transduced with herpes simplex virus amplicons encoding human immunodeficiency virus type 1 (HIV-1) gp120 elicit adaptive immune responses from human cells engrafted into NOD/SCID mice and confer partial protection against HIV-1 challenge. *J Virol*. 2005; 79:2124–2132. [PubMed: 15681415]

34. Gorantla S, Sneller H, Walters L, Sharp JG, Pirruccello SJ, West JT, et al. Human immunodeficiency virus type 1 pathobiology studied in humanized BALB/c-Rag2^{-/-} gammac^{-/-} mice. *J Virol*. 2007; 81:2700–2712. [PubMed: 17182671]
35. Cota M, Kleinschmidt A, Ceccherini-Silberstein F, Aloisi F, Mengozzi M, Mantovani A, et al. Upregulated expression of interleukin-8, RANTES and chemokine receptors in human astrocytic cells infected with HIV-1. *J Neurovirol*. 2000; 6:75–83. [PubMed: 10786999]
36. Gorantla S, Makarov E, Finke-Dwyer J, Gebhart CL, Domm W, Dewhurst S, et al. CD8⁺ cell depletion accelerates HIV-1 immunopathology in humanized mice. *J Immunol*. 2010; 184:7082–7091. [PubMed: 20495069]
37. Hamblin MR, Miller JL, Rizvi I, Loew HG, Hasan T. Pegylation of charged polymer-photosensitizer conjugates: effects on photodynamic efficacy. *Br J Cancer*. 2003; 89:937–943. [PubMed: 12942129]
38. Fredericksen BL, Wei BL, Yao J, Luo T, Garcia JV. Inhibition of endosomal/lysosomal degradation increases the infectivity of human immunodeficiency virus. *J Virol*. 2002; 76:11440–11446. [PubMed: 12388705]
39. Miyauchi K, Kozlov MM, Melikyan GB. Early steps of HIV-1 fusion define the sensitivity to inhibitory peptides that block 6-helix bundle formation. *PLoS Pathog*. 2009; 5:e1000585. [PubMed: 19763181]
40. Miyauchi K, Kim Y, Latinovic O, Morozov V, Melikyan GB. HIV enters cells via endocytosis and dynamin-dependent fusion with endosomes. *Cell*. 2009; 137:433–444. [PubMed: 19410541]
41. Sharma NR, Mateu G, Dreux M, Grakoui A, Cosset FL, Melikyan GB. Hepatitis C virus is primed by CD81 protein for low pH-dependent fusion. *J Biol Chem*. 2011; 286:30361–30376. [PubMed: 21737455]
42. Meertens L, Bertaux C, Dragic T. Hepatitis C virus entry requires a critical postinternalization step and delivery to early endosomes via clathrin-coated vesicles. *J Virol*. 2006; 80:11571–11578. [PubMed: 17005647]
43. Blanchard E, Belouzard S, Goueslain L, Wakita T, Dubuisson J, Wychowski C, et al. Hepatitis C virus entry depends on clathrin-mediated endocytosis. *J Virol*. 2006; 80:6964–6972. [PubMed: 16809302]

Fig. 1.
(A) Schematic representation of spontaneous formation of APN as a result of electrostatic coupling of the cationic peptide and anionic block copolymer. **(B)** Tapping-mode AFM images of (left) p41/PEG-PLD₂₀ APN and (right) p41. Scan size is 2 μm . The insert shows an image of an individual APN particle. Bar equals 30 nm. Samples were deposited onto (left) positively charged APS-modified mica or (right) freshly cleaved negatively charged mica.

Fig. 2. CD spectra of p41 (dashed line), PEG-PLD₂₀ copolymer (dotted line), and APN (p41/PEG-PLD₂₀) (solid line) recorded in 10 mM phosphate buffer, pH 7.4, at 20°C. The peptide concentrations were 100 μM . Complexes were prepared at $Z_{-/ +} = 1$.

Fig. 3.

APN displayed enhanced stability against proteolytic degradation. MALDI-TOF spectra represent intact p41 and APN and inserts represent spectra of digested p41 and APN after incubation on trypsin column for 13 minutes. Spectra show the existence of intact p41 with the peak at 2433 (m/z) along with the p41 fragments (561 and 474) in APN sample, whereas p41 was completely degraded. Red ovals indicate non-digested p41 and arrows show the protected p41 after APN digestion.

Fig. 4. Hemolytic activity of APN and its constituents as a function of concentration. APN, peptides, or copolymer at different concentrations (peptide equivalents) were mixed with human RBC for 1 h in isotonic solution and hemolytic activity was normalized to positive control, 1% v/v Triton X-100 solution, as a 100% hemolysis. Data presented as mean $\pm$ SD (n = 3).

Fig. 5. Uptake of APN in Huh7.5 cells. Cell cultures were exposed to 10 μ M concentrations of p41-Cy5 and APN (p41-Cy5/PEG-PLD₂₀) for 1, 2 and 4 h, washed, fixed and confocal images were captured at 40 $\times$ objective. Red staining corresponds to p41-Cy5, cell nuclei were stained with DAPI (blue). Apoptotic cells are indicated by white arrows.

Fig. 6. APN uptake by macrophages. **(A)** The time-dependent uptake of APN and PEG-PLD₂₀ by MDM in suspension was determined by intracellular staining with rabbit monoclonal antibodies against PEG followed by FACS analysis. p41-Cy5 uptake was measured by direct fluorescence. **(B, C)** Detection of APN uptake by attached MDM culture by staining for PEG (red) and co-localization with earlier endosomal antigen 1 (EEA1, green) at 2 hours of incubation. Cell nuclei were stained with DAPI (blue). The set of images consists of a single color and overlay **(B)** and a side view of the XZ optical line scan through the red line, and a side view of the YZ optical line scan through the green line **(C)**. ON side view optical image, yellow color indicates PEG colocalized with EEA1-stained vesicles. Magnification is at 63×/1.4 Oil DIC M27. Scale bar 10 μ m.

Fig. 7.

APN anti-HCV activity in *vitro*. **(A)** Intracellular HCV RNA after 1 h pretreatment of Huh7.5 cells with p41 and APN at a dose equivalent to 10 μ M peptide prior HCV infection. APN demonstrated enhanced ability to suppress HCV infection. Data presented as mean $\pm$ SD (n = 3); * - $p < 0.05$. **(B)** FACS analysis of Huh7.5 cells pretreated for 1 h with APN at various peptide concentrations before infection with following 3 additional treatments during culture medium exchange. APN significantly suppressed HCV core antigen expression.

Fig. 8.

APN suppress HIV-1 replication in cell cultures. (A) Pretreatment of TZM-bl cells for 2 h before HIV-1_{ADA} infection with p41 showed reduction of the number of infected cells. Protective effects of APN were observed even at the lower concentrations of peptide. (B) APN pre-treatment exhibited superior protection of PHA-activated human lymphocytes from HIV-1_{ADA} infection compared to p41 and p1. (C) APN exhibited the most potent anti-viral activity and suppressed the ongoing HIV infection. MDM were pre-treated for 2 h, HIV-1_{ADA} infected overnight, washed and additional treatment with APN and peptides at a dose equivalent to 10μM peptide were performed every other day. RT activity was adjusted

to MTT values/well. Data presented as mean $\pm$ SD (n = 4); * - $p < 0.05$ statistically significant differences between p41 and APN.

Fig. 9.

Anti-HIV activity of APN *in vivo*. NSG mice were transplanted with hu-PBL and 7 days later i.p. inoculated with HIV-1_{ADA} 10⁴ TCID₅₀. In 30 minutes prior virus inoculation mice were injected i.m. with 50μg of free p41 or its APN format. Animals were then treated with p41 or APN on daily basis for the next 6 days, and were euthanized on day 7 post infection. The uninfected (open bars) and HIV-infected (black bars) animals with similar engraftment of human cell (CD45+ cell % in spleen, **A**) were compared for CD4+ and CD8+ T cell numbers (**B**, **C**), CD4:CD8 ratios (**D**) and *HIVgag* RNA tissue expression (**E**). APN treatments significantly protected CD4+ cells and suppress *HIVgag* expression in spleen

tissue. Gray lines in panel E represent median of viral RNA normalized to GAPDH values for treated groups of animals ($n = 6$). * - $p < 0.01$, # - $p < 0.03$, ## - $p < 0.004$ compared to HIV-infected control animals.

Table 1

Physicochemical characteristics of block ionomers

Copolymer ^a	Molecular weight	Polydispersity index	Average number of units in ionic block ^a
PEG-PLD ₁₀	6,400	1.03	10
PEG-PLD ₂₀	7,200	1.05	20
PEG-PLE ₁₀	6,500	1.15	10
PEG-PLE ₂₅	8,800	1.2	25
PEG- PLE ₄₀	11,000	1.01	40

^aThe average molecular weights, polydispersity indices and the average number of monomer units in polyacid blocks were provided by manufacturer. The molecular weight of PEG block was 5,000.

Table 2Physicochemical characteristics of APN^a

Sample	D _{eff} (nm) ^b	PDI ^b
p41	902	0.785
p41/PEG-PLD ₁₀	32.9 ± 3.5	0.14
p41/PEG-PLD ₂₀	36.5 ± 5.5	0.12
p41/PEG-PLE ₁₀	31.1 ± 1.7	0.09
p41/PEG-PLE ₂₅	43.4 ± 6.1	0.17
p41/PEG-PLE ₄₀	71.1 ± 6.9	0.24

^aComplexes were prepared in PBS (pH 7.4, 0.14 M NaCl) at Z₋/Z₊=1.^bEffective diameter (D_{eff}) and polydispersity indices (PDI) were determined by DLS at 25°C (n=3).