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The influence of charge clustering on the anti-HIV-1 activity and *in vivo* distribution of negatively charged albumins

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Abstract

The substitution of human serum albumin with negatively charged molecules, such as succinic acid (Suc–HSA) or aconitic acid (Aco–HSA), resulted in proteins with potent anti-HIV activities, by binding to viral gp120 (V3 loop). The aim of the present study was to investigate whether the distribution of negative charges on the albumin backbone influences the anti-HIV activity. Therefore, we prepared albumins with clusters of negatively charged groups by coupling of heparin. The effects of this substitution on anti-HIV activity, *in vivo* distribution and the protein structure as compared to random succinylation were assessed. *In vitro* studies indicated that HSA-modified with heparin 6 or 13 kD displayed anti-HIV activity (IC_{50} = 660 and 37 nM, respectively) and exhibited affinity for gp120-V3 loop, although the activity was lower than that of Suc–HSA. Combined derivatization of HSA with heparin 13 kD and aconitic acid groups resulted in significantly increased inhibitory actions (IC_{50} = 2.8 nM). Structural analysis showed that modification of HSA with heparin did not lead to extensive unfolding of the protein, meaning that these modified proteins were still globular in structure. In contrast, succinylation of HSA resulted in a highly randomly coiled conformation. Dynamic light scattering experiments revealed that, at neutral pH, the heparin fragments attached to the protein were wrapped around the molecule rather than sticking out into the solution. In conclusion, coupling of sufficient clustered negative charges, by coupling of Hep-fragments, on HSA resulted in a clear anti-HIV activity of the protein. Yet, random distribution of anionic groups in the albumin seemed more optimal for *in vitro* anti-HIV activity. The higher plasma and lymphatic concentrations of Hep–HSA compared to Suc–HSA seemed more favorable for an anti-HIV activity *in vivo*.

Keywords: HIV; Polyanions; Suc–HSA; Structure; Heparin; Entry inhibitor

1. Introduction

The first agents developed with therapeutic activities against HIV were directed against the viral enzymes reverse transcriptase and protease. The development of viral strains that are resistant against these agents has continued the search for compounds exerting anti-HIV activities at other levels of the viral replication cycle. Furthermore, drugs are needed that are less toxic and/or that exert anti-HIV activities in latent viral reservoirs. A new class of anti-HIV drugs are agents that interfere with the cellular entry of HIV [1,2]. Various stages in the binding of HIV to host cells are of interest for therapeutic intervention. Of these, interference by binding to one of the envelope proteins of the virus (gp120 and gp41), or binding to one of the cell membrane receptors involved in the binding of HIV particles (CD4 receptor or chemokine

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Abbreviations: HSA, human serum albumin; Suc–HSA, HSA-modified with succinic acid; Aco–HSA, HSA-modified with aconitic acid; Hep–HSA, HSA-modified heparin moieties; pI, isoelectric point; Hep, heparin; IC_{50}, 50% inhibitory concentration; CC_{50}, 50% cytotoxic concentration; i.v., intravenous; ^{1}H-NMR, nuclear magnetic resonance; DSC, differential scanning calorimetry; DLC, dynamic light scattering.

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co-receptors CXCR4 and CCR5) are optional. This class of anti-HIV drugs may complement those that interfere with other phases in the viral replication process. Advantages of combining such entry blockers with agents that inhibit later stages in the viral replication process could be a reduced occurrence of resistance and the potential synergistic effects [1,2].

Polyanionic compounds such as heparin and dextran sulfate are potent inhibitors of the cellular entry of HIV [3]. These polysulfates exert their effects by binding to the V3 region in gp120 [4]. However, the antiviral activities observed in clinical trials were disappointing. In addition, the infusion of these agents proved to be toxic due to, amongst others, thrombocytopenia [5].

Still, blocking of the V3 loop seems to be a promising mode of action, as the V3 loop plays a critical role in the CD4 dependent and independent attachment of HIV to cells [6]. In our laboratory, negatively charged proteins have been developed that exert potent anti-HIV activities. The antiviral properties of these negatively charged proteins, i.e. Suc–HSA or Aco–HSA [7,8], are based on binding to the virus particles, in particular to the V3 loop and C-terminal part of gp120, which are both positively charged [9,10]. In contrast to the polysulfates, these negatively charged albumins displayed no anticoagulant activity [11], are biodegradable, and proved to be non-toxic in rats and monkeys [12,13]. Other negatively charged proteins that were studied for their anti-HIV activities are milk proteins, such as β-lactoglobulin or casein modified with 3-hydroxypthalic acid [14,15]. Although potent anti-HIV activities were measured, the 3-hydroxyphtalic acid derivatives also display cytotoxic activities, because these agents were not chemically stable during long term culture [14].

The binding between gp120 and the negatively charged albumins may be influenced by the anionic charge distribution as well as hydrophobic parts in the negatively charged albumin. In the present study, we studied the structural elements that are required for an optimal anti-HIV activity in more detail. Previously, Swart *et al.* [8] assessed that the charge density on a protein backbone was of importance for anti-HIV activity. The aim of this underlying study was to determine whether a general (at random) distribution of negative charges on the albumin molecule is optimal for anti-HIV activity or that attachment of clustered anionic charges would also be effective. Therefore, we prepared albumins linked to various small molecular weight heparins, introducing clusters of negative charges on the protein, and assessed *in vitro* the anti-HIV potency (IC_{50}) and binding affinity for the V3 loop. Since the *in vivo* behavior of compounds will affect the antiviral effects *in vivo*, we also studied their pharmacokinetic profile in rats. Furthermore, we examined whether the type of protein modification (acylation or heparinization) influenced the structure of the backbone protein.

2. Materials and methods

2.1. Reagents and chemicals

HSA, consisting of at least 95% monomeric protein, was purchased from the Central Laboratory of the Blood Transfusion Services (Sanguin). Heparin 3, 6 and 13 kD were obtained from Sigma. All other chemicals used were of analytical grade.

Modified albumins were labeled with ^{125}I using the chloramine-T method [16]. Prior to each experiment non-covalently bound ^{125}I was removed by dialysis against phosphate buffered saline pH 7.4 (PBS) to obtain preparations with less then 5% free ^{125}I as determined by precipitation with 10% trichloro-acetic acid (TCA) containing 0.1% NaI.

Also, proteins were labeled with FiTC (fluorescein isothiocyanate, Sigma) as described [11].

2.2. Synthesis of negatively charged albumins

2.2.1. Suc–HSA and Aco–HSA

HSA was substituted with succinic acid or aconitic acid groups and the products were characterized as described previously [8].

2.2.2. Hep–HSA

Heparin fragments of various chain lengths (3, 6 and 13 kD) were coupled to the lysine ε-NH_2 groups of HSA according to the following protocol. First, heparin (20 mg/mL) was treated with $NaNO_2$ (0.2 mg/mL) at pH 2.7 to yield a reactive aldehyde group. After 16 hr of incubation at room temperature, the reaction mixture was added to an HSA solution (62.5 mg/mL 0.2 M phosphate buffer pH 7.4). Ten milligram per milliliter $NaBH_3CN$ (Sigma) was added and the reaction was allowed to proceed at room temperature for 4 days. A fresh portion of $NaBH_3CN$ (5 mg/mL) was added at day 3. Hep–HSA conjugates were separated from unreacted HSA and heparin by HiPrep 16/10 DEAE Sepharose and HiTrap Blue column chromatography (both Amersham Pharmacia). Finally, the Hep–HSA fractions were dialyzed against water, lyophilized, and stored at -20°.

2.2.3. Aconytilation of Hep–HSA

Hep–HSA was modified with additional aconitic acid groups as described above for HSA [8].

2.3. Characterization of Hep–HSA

The molecular weight of the $NaNO_2$-treated heparin fragments was assessed by FPLC (Fast Protein Liquid Chromatography) analysis. The reaction between heparin and $NaNO_2$ was stopped after 16 hr of incubation by adjusting the pH to 7 with 0.1N NaOH. The samples were then loaded on a Superdex 75 column (Amersham Pharmacia)

and eluted with PBS at a flow rate of 0.5 mL/min. The heparin fractions were identified with UV detection at 214 nm.

All lyophilized Hep–HSA preparations were characterized by determination of the protein content (A280 nm), the degree of modification as assessed by determination of free amine groups present in the modified albumins [17], the content of heparin per HSA molecule by reaction with azure A [18], and by polyacryl amide gel electrophoresis (7.5%, -SDS) followed by colloidal coomassie brilliant blue (protein) or Azure A (heparin) staining [19].

The pI of the preparations were determined with the FPLC system equipped with a MonoP column (Amersham Pharmacia) as described by Burness *et al.* [20], with slight modifications. The column was equilibrated before each run with buffer A (25 mM piperazine pH 5.7). After a 6 mL pre-gradient with buffer B (Polybuffer 74 diluted 1:8; Amersham Pharmacia Biotech), 1 mg/mL sample was injected and eluted with a gradient of successively 20 mL buffer B, 25 mL buffer C (25 mM formic acid pH 4), and 15 mL buffer D (25 mM oxalic acid pH 1), at a flow rate 0.25 mL/min. Fractions of 1 min were collected. The pH and the protein concentration (Biorad) of these fractions were determined. The pI of the preparation was set at the pH measured in the fraction with the highest protein concentration.

The stability of the Hep–HSA preparations was determined after incubation of 10 mg/mL protein at 37° for various time periods ($t = 0$, 0.5, 1, 2, 4, 24, 48 hr). Incubations were performed in PBS pH7.4 and in rat serum. Release of heparin fragments were assessed with polyacryl amide gel electrophoresis (12% gel, -SDS) and Azure A (Hep) staining [19].

2.4. Structure analysis of negatively charged albumins

2.4.1. Nuclear magnetic resonance

^{1}H-NMR spectra were obtained at 25° using a Bruker AM400 spectrometer operating at 400.13 MHz, connected to an Aspect 3000 computer. Protein samples were dissolved in PBS (made in D_2O) for the ^{1}H-NMR experiments, except for Suc–HSA, which was dissolved in D_2O. The concentration of the proteins was determined using the intensity of the resonances of protons of the $-CH_3$ groups from Leu, Val and Ile (resonances between 0.8–1.1 ppm) that were obtained from NMR spectra of the protein samples which were denaturated using 8 M deuterated urea [21]. For the concentration determinations a mixture of 3-trimethylsilyl propionic acid-d4 (TSP) (Fluka), 2,2,2-triflouroethanol (Aldrich) and methanol (Baker) in D_2O were used as standards. The ^{1}H-NMR spectra were calibrated using the D_2O resonance.

2.4.2. Differential scanning calorimetry

The heat-induced conformational changes of the various (modified) proteins was studied by DSC using a Perkin-Elmer DSC7 apparatus. Samples were scanned at a rate of 10°/min in the temperature range of 20–110°.

2.4.3. Dynamic light scattering

DLS experiments were performed using a Spectra Physics 275 mW Ar laser (514.5 nm) and a digital ALV-5000 correlator. The sample housing was kept at 20° during the experiments. The detector of the DLS was set at an angle of 90°. Prior to the measurements, the protein solutions (2 mg/mL, in double distilled water) were filtered through a low protein binding filter (0.22 μm Millex-VV, Millipore). We analyzed the data using a double exponential fit.

2.5. Anti-HIV activity in vitro

1. Screening of the anti-HIV-1 activity of the preparations was performed at Tibotec as described [22]. Briefly, MT4 cells were either infected with HIV (strain IIIb) or mock-infected. Five days after infection the viability of the mock- and HIV-infected cells was examined by MTT method. The IC_{50} was defined as the concentration of the compound that protected HIV infected cells by 50%, whereas the CC_{50} was defined as the concentration that reduced the viability of mock-infected cells by 50%. The selectivity index (SI) was defined as the ratio between CC_{50} and IC_{50}.
2. We also tested the effect of 10 μM modified albumin on the spreading of cellular infection *in vitro*. For this study, the primary HIV isolate LAI and the SupT1 T-cell line were used. CA-p24 production in the culture supernatant was measured by ELISA as described [23].

2.6. Binding to gp120-V3 loop

Binding of the Hep–HSA preparations to the V3 loop of gp120 was assessed by incubating the proteins with V3 loop peptides according to Kuipers *et al.* [9]. The linear peptides V3-160bal (TRK**S**IHIGPGRAFYTTG**E**IIG**D**IRQAHC), which represents the NSI (non syncytium inducing) HIV type, and V3-Q17 (TRK**R**IHIGPGRAFYTTG**Q**IIG**N**IRQAHC), which represents the SI (syncytium inducing) variant, were prepared by Eurosequence. The peptides were immobilized by coupling to CNBR-activated Sepharose (Pharmacia) and incubated with ^{125}I-labeled modified albumin for 3 hr at room temperature. After washing, the total amount of ^{125}I-modified albumin bound to the peptide-Sepharose beads was assessed with a γ-counter. For the inhibition studies, a constant amount of ^{125}I-labeled protein (±80,000 cpm) was diluted with various concentrations of non-labeled inhibitor (0.001–100 μg) and the experiments were performed as described before, using 5 μg V3Q17 peptide coupled to Sepharose beads in these assays.

2.7. Pharmacokinetic behavior of Hep–HSA in vivo

2.7.1. Animals

Male Wistar rats (Harlan), weighing ±250 g, were housed in a temperature-controlled room with a 12 hr light/dark regimen and free access to water and food. The study as presented was approved by the Local Committee for Care and Use of Laboratory Animals.

2.7.2. Organ distribution

After anesthesia with Hypnorm/diazepam, a tracer dose (10 ng, 1.10^6 cpm) of the ^{125}I-modified albumins was injected i.v. *via* the penis vein. Ten minutes after administration, blood samples were taken by heart puncture and the rats were sacrificed. Subsequently, tissues were removed, washed in saline, and analyzed for the presence of radioactivity. The total radioactivity per organ was calculated with a correction for blood-derived radioactivity in the particular organs, as determined previously using ^{125}I-HSA [24].

2.7.3. Cellular localization

Anaesthetized rats received an i.v. bolus dose of 4 mg/kg Hep–HSA (FiTC labeled). Ten minutes after injection, various organs were removed and snap frozen in isopentane (−80°). Cryostat sections (4 μm) were made, fixed with acetone, and stained with a mouse monoclonal antibody directed against FiTC (DAKO) or rabbit polyclonal anti-HSA antibody (ICN) for the localization of modified albumins using an indirect immunoperoxidase method. Of note, free FiTC is not detected, because FiTC alone will not be fixed in tissue sections.

2.7.4. Plasma and lymphatic concentrations of modified HSA in time

After anesthesia with Hypnorm/diazepam, the thoracic duct and carotid artery were cannulated to collect lymph and blood, respectively [25,26]. The proteins injected were labeled with either FiTC or ^{125}I.

A bolus dose (4 mg/kg, dissolved in PBS) Hep–HSA or Suc–HSA was i.v. injected *via* the penis vein. Blood and lymph samples were collected at indicated time points between 2 and 120 min after administration. Plasma and lymphatic concentrations of modified albumin were determined using a γ-counter or fluorimeter. Pharmacokinetic analysis of the plasma concentration–time data was performed with MultiFit software for non-linear curve fitting (J.H. Proost, University Center for Pharmacy) using the population analysis Bayesian iterative two-stage.

2.8. Anticoagulant activity of heparin modified proteins

Different concentrations of the modified albumins (1, 10 and 100 μg/mL) were spiked to pooled human plasma. The samples were assayed for their anticoagulant activity by the activated partial thromboplastin time (APTT) and the anti-Xa assay, using routine clinical analysis (Academic Hospital Groningen). Heparin 3 and 13 kD were used as positive controls, whereas unmodified HSA served as a negative control.

2.9. Statistical analysis

All data are expressed as means ± SD. Statistical analysis was performed by using an unpaired two-tailed Student's *t*-test. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Synthesis and characterization of Hep–HSA preparations

A clustering of negative charges on albumin was obtained by covalent coupling of heparin fragments to lysine NH_2-groups in HSA. To prepare compounds with a varying number of clustered charged groups, various chain lengths of heparin (i.e. 3, 6 and 13 kD) were attached.

In the heparin molecule, an active aldehyde group should be generated for covalent substitution of HSA, which was achieved by reaction with $NaNO_2$. This treatment only slightly reduced the heparin chain length as assessed with FPLC Sephadex 75 chromatography. The retention time for Hep3 kD shifted from 33.3 to 33.9 min, for Hep6 kD from 29.6 to 31.7 min, whereas for Hep13 kD it increased from 23.3 to 25.1 min (chromatograms not shown).

The coupling of the heparin fragments to the lysine residues in HSA resulted in preparations that varied either in length of the anionic fragments or in the degree of albumin substitution (see Table 1). In general, when shorter Hep-chains were coupled to HSA more lysine NH_2-groups were substituted. The covalent attachment of heparin groups to HSA was confirmed by serial staining of the gels for protein (with coomassie brilliant blue) and heparin (with Azure A). Also gel electrophoresis showed that the products were predominantly in the monomeric form (>90%) (data not shown). The Hep–HSA preparations had a pI value similar to HSA-modified with succinic or aconitic anhydride, which is an indication that the derivatized proteins were about equally negatively charged. Furthermore, we combined the clustered and random substitution by coupling of aconitic acid groups to Hep13 kD–HSA. This resulted in the protein Aco-Hep13 kD–HSA that also displayed strongly negatively charged properties (pI 2.4, Table 1).

The coupling of heparin to the HSA backbone resulted in a covalent binding between these compounds. After incubation of the preparations at 37° in PBS and in serum, up to 48 hr, no free heparin could be detected. The detection limit of free heparin with this method was ±50 ng. The

Table 1
Chemical characterization of the various negatively charged albumins

Compound	Protein content (%)	Heparin content (μg Hep/mg HSA)	No. of occupied NH_2 groups	pI
HSA	100	–	–	5.2
Suc–HSA	94	–	60	2.5
Aco–HSA	75.9	–	52	2.4
Hep3 kD–HSA	97.4	34.3	10	2.5
Hep6 kD–HSA	87.5	91.2	9	2.5
Hep13 kD–HSA-I[a]	92.9	86.9	3	2.4
Hep13 kD–HSA-II[a]	95.8	150.3	12	–
Aco–Hep13 kD–HSA	–	150.3	60	2.4

[a] Two preparations of Hep13 kD–HSA that differ in number of Hep coupled to HSA.

Azure A staining in the gel was detected in all cases only at the HSA level, indicating that heparin moieties remained attached to protein backbone.

3.2. Structure of the negatively charged proteins

3.2.1. ^{1}H-NMR

In general, (surface) modification affects the structure of the protein molecule due to both the bulkiness of the chemical groups that are introduced on the protein and the concomitant changes in the local charges by the modifications. The overall protein fold may be affected by such modifications, which may even result in the unfolding of the protein [14]. In order to get insight in the effect of the modifications by heparin, the proteins were studied by ^{1}H-NMR. Fig. 1 shows the ^{1}H-NMR spectra of various (modified) HSA preparations. Also the ^{1}H-NMR spectrum of HSA denatured in 8 M urea is shown. Comparing the ^{1}H-NMR spectra of native HSA with that of HSA in 8 M urea clearly shows that denaturation of HSA has a dramatic effect on its ^{1}H-NMR-spectrum. In the aromatic region (6.5–8 ppm) much less chemical dispersions was observed. A similar effect was visible in the aliphatic region of the ^{1}H-NMR spectra (0–4 ppm), where the spectrum showed a clear random coil pattern. This is mainly due to the resonances of the methyl groups of Val, Leu and Ile. As a result of the unfolding, the protein adopts a more random coil-like structure resulting in much sharper resonance lines in the ^{1}H-NMR spectra. From this figure it is also evident that the ^{1}H-NMR spectra of Hep13 kD–HSA and Hep3 kD–HSA are similar to that of the native molecule. Suc–HSA, however, displayed a spectrum that is similar to that of denatured HSA. This indicates that succinylation had a severe effect on the overall structure of HSA, and resulted in unfolding of the protein molecule.

3.2.2. DLC

Temperature dependent denaturation of the native HSA, Suc–HSA, Hep13 kD–HSA and Hep3 kD–HSA was studied with DSC. In these experiments, typical endothermic energy changes were observed for all protein preparations except for Suc–HSA, where no endothermic energy change could be seen (Table 2). For native HSA the energy change (Td_{app}) occurs around 77°, whereas for Hep3 kD–HSA and Hep13 kD–HSA broader peaks in the DSC thermograms with maxima around 70° were seen. Since the Hep–HSA preparations consisted of a mixture of different degrees of modification broader thermograms were expected with a

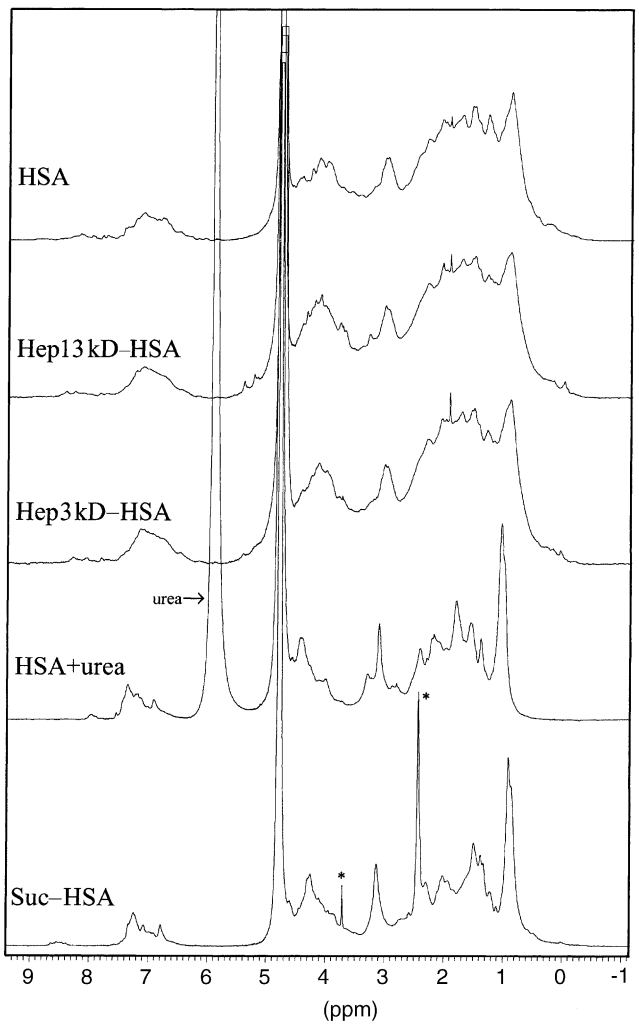


Fig. 1. ^{1}H-NMR spectra of HSA, Hep3 kD–HSA, Hep13 kD–HSA, HSA in 8 M urea and Suc–HSA. Spectrum 1: native HSA; spectrum 2: Hep13 kD–HSA; spectrum 3: Hep3 kD–HSA; spectrum 4: HSA in 8 M urea; spectrum 5: Suc–HSA. (*): unidentified peak; the resonance belonging to urea is indicated.

Table 2
Thermodynamic parameters for the thermal denaturation of HSA, Hep13 kD–HSA, Hep3 kD–HSA and Suc–HSA

	ΔH_{app} (J/g)	Td_{app} (°)
HSA	13	77
Hep13 kD–HSA^{-I}	2.1	70
Hep3 kD–HSA	9.3	68
Suc–HSA	nd[a]	–

[a] nd: no endothermic energy change was detected.

different apparent temperature maximum of the observed energy change. However, the total heat change observed for HSA and for the heparin modified proteins were similar. These observations are in line with the NMR experiments showing that the modification with heparin hardly affected the overall protein structure. In contrast, (complete) succinylation of HSA had an enormous impact on the protein structure, resulting in unfolding of the molecule. As a result no heat effect was observed in the DSC experiments, which is also in line with the NMR-experiments.

3.2.3. DLS

The modification of HSA with heparin will result in 'clusters' of negative charge on the surface of the protein. Since these polymers are covalently attached to HSA, they may either protrude into the solution or they may interact with the protein surface in such a way that they are wrapped around the protein molecule, covering the protein surface. DLS is a technique that is sensitive to the hydrodynamic particle size of a protein. Upon attachment of heparin molecules to a protein surface one would expect a large effect (5–6 nm for Hep3 kD and 12–16 nm for Hep13 kD) on the effective hydrodynamic particle size if these heparin polymers were completely sticking into the solution. If the polymers are wrapped around the molecule a relatively small increase in hydrodynamic radius is expected relative to that of the native protein (about 1–2 nm for 3 kD heparin and about 2–3 nm for 13 kD heparin). The particle sizes (i.e. hydrodynamic radius) measured by dynamic light scattering of HSA, Hep3 kD–HSA and Hep13 kD–HSA were 2.7, 3.7 and 5.3 nm respectively. These small increases in hydrodynamic radius upon modification showed that the heparin molecules attached to the protein surface are interacting with the protein surface, such that the heparin polymers are in fact 'wrapped' around the protein molecule.

3.3. Anti-HIV activity in vitro

The anti-HIV-1 (IIIb) activity of the heparin fragments and negatively charged albumins are summarized in Table 3. In general, HSAs modified with Hep-fragments were less potent inhibitors of HIV replication compared with HSA-modified by random succinylation or aconitylation, as reflected by the higher IC_{50} values. HSA-modified with Hep3 kD did not show activity at all, in contrast to the albumins modified with Hep6 kD and Hep13 kD. Interestingly, attachment of aconitic acid groups to Hep13 kD–HSA resulted in an increased anti-HIV activity that was comparable to the IC_{50} of Suc–HSA and Aco–HSA (Fig. 2). We also measured the antiviral activity of the various unconjugated heparin fragments. Table 3 shows that Hep3 kD is less active than heparins of 6 or 13 kD length. The treatment of Hep with $NaNO_2$, necessary for its covalent coupling to HSA, resulted in a decrease in antiviral activity. From these data, it can be concluded that coupling of heparin to HSA did not improve the anti-HIV activity as compared to uncoupled heparin. None of the Hep–HSA preparations exerted cytotoxic effects *in vitro* up to the maximum concentration studied. A possible toxicity of the compounds *in vivo* remains to be studied.

Table 3
Anti-HIV-1 (strain IIIb) activity (IC_{50}) and cytotoxicity (CC_{50}) of the negatively charged albumins and uncoupled heparin controls

Compound	IC_{50} (μg/mL)	IC_{50} (nM)	CC_{50} (μg/mL)	SI
HSA	>250	>3731	>250	>1
Suc–HSA	0.11	1.57	>250	>2273
Hep3 kD–HSA	>250	>2660	>250	>1
Hep6 kD–HSA	79.89	660	>250	>3.13
Hep13 kD–HSA-I	20.18	190	>250	>12.4
Hep13 kD–HSA-II	5.36	36.9	>250	>46.6
Aco–Hep13 kD–HSA	0.32	2.8	>250	>781
Aco_{52}–HSA	0.037	0.49	>250	>6757
Hep3 kD	4.65	1550	>250	>53.8
Hep3 kD ($NaNO_2$)[a]	38.20	8682	>250	>6.5
Hep6 kD	0.73	121	>250	>343
Hep6 kD ($NaNO_2$)[a]	0.82	137	>250	>305
Hep13 kD	0.86	66.5	>250	>291
Hep13 kD ($NaNO_2$)[a]	3.63	279	>250	>68.9

[a] Anti-HIV activity of Hep treated with $NaNO_2$.

HSA and Hep3 kD–HSA also did not exert inhibitory effects sin another *in vitro* anti-HIV assay, in which replication of the LAI isolate in the SupT1 T-cell line was

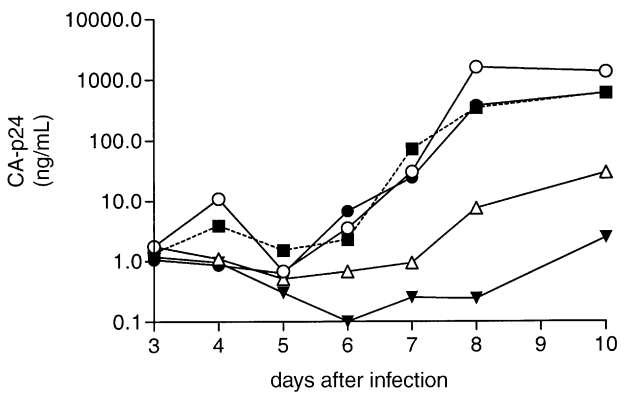


Fig. 2. Anti-HIV activity of Suc–HSA (–▼–), Hep3 kD–HSA (–○–), Hep13 kD–HSA (–△–) and HSA (–●–) as compared to a control infection (···■···) of SupT1 T-cells with the LAI isolate. Virus replication was monitored by CA-p24 measurement in the culture supernatant.

followed for up to 10 days (Fig. 1). In contrast, Hep13 kD–HSA^{-I} and, in particular, Suc–HSA significantly reduced viral replication. The culture contained 600 ng/mL CA-p24 in the control situation, whereas in the presence of 10 μM Suc–HSA or Hep13 kD–HSA significantly reduced levels of CA-p24 were produced (2.4 and 10 ng/mL, respectively).

3.4. Binding to V3 loop of gp120

The binding of the modified albumins (^{125}I-labeled) to the V3 region of gp120 was determined with linear V3 peptides (Fig. 3A). The randomly modified protein Suc–HSA showed highest binding to the V3 peptides, whereas the control protein HSA did not have affinity for the V3 loop. These results corroborate previous data described by Kuipers *et al.* [9]. However, we were only able to measure binding of the negatively charged proteins to the SI variant (V3-Q17) and not to the NSI variant (V3-160bal) of the V3 loop (data not shown). Hep13 kD–HSA^{-I} exerted a significant interaction with V3 peptides, although to a lesser extent than Suc–HSA. The binding to V3 peptides decreased when shorter chain lengths of heparin were coupled to HSA. Furthermore, Hep13 kD–HSA^{-I} was able to interfere with the binding of ^{125}I-labeled Suc–HSA to V3-Q17, indicating binding of both proteins to the same epitope (Fig. 3B). Suc–HSA and Hep13 kD–HSA both inhibited ^{125}I-Suc–HSA–V3-Q17 interaction dose dependently, whereas the other heparin modified albumins (Hep3 kD–HSA and Hep6 kD–HSA) and HSA itself did not affect this binding to a statistically significant extent.

To study the V3 loop interaction of Suc–HSA and Hep13 kD–HSA in more detail, we incubated the ^{125}I-labeled proteins with increasing concentrations of unlabeled Suc–HSA and Hep–HSA (Fig. 3C and D). Both proteins were best inhibited by their own, and to a somewhat lesser extent with the other negatively charged compound. The amount of protein that causes a 50% reduction of the total radioactive binding was 0.3 μg Suc–HSA and 3 μg Hep13 kD–HSA in case of ^{125}I-Suc–HSA. In case of ^{125}I-Hep13 kD–HSA, 0.7 μg Hep–HSA or 4 μg Suc–HSA was needed. This indicated that both modified albumins showed a comparable affinity for the V3 loop.

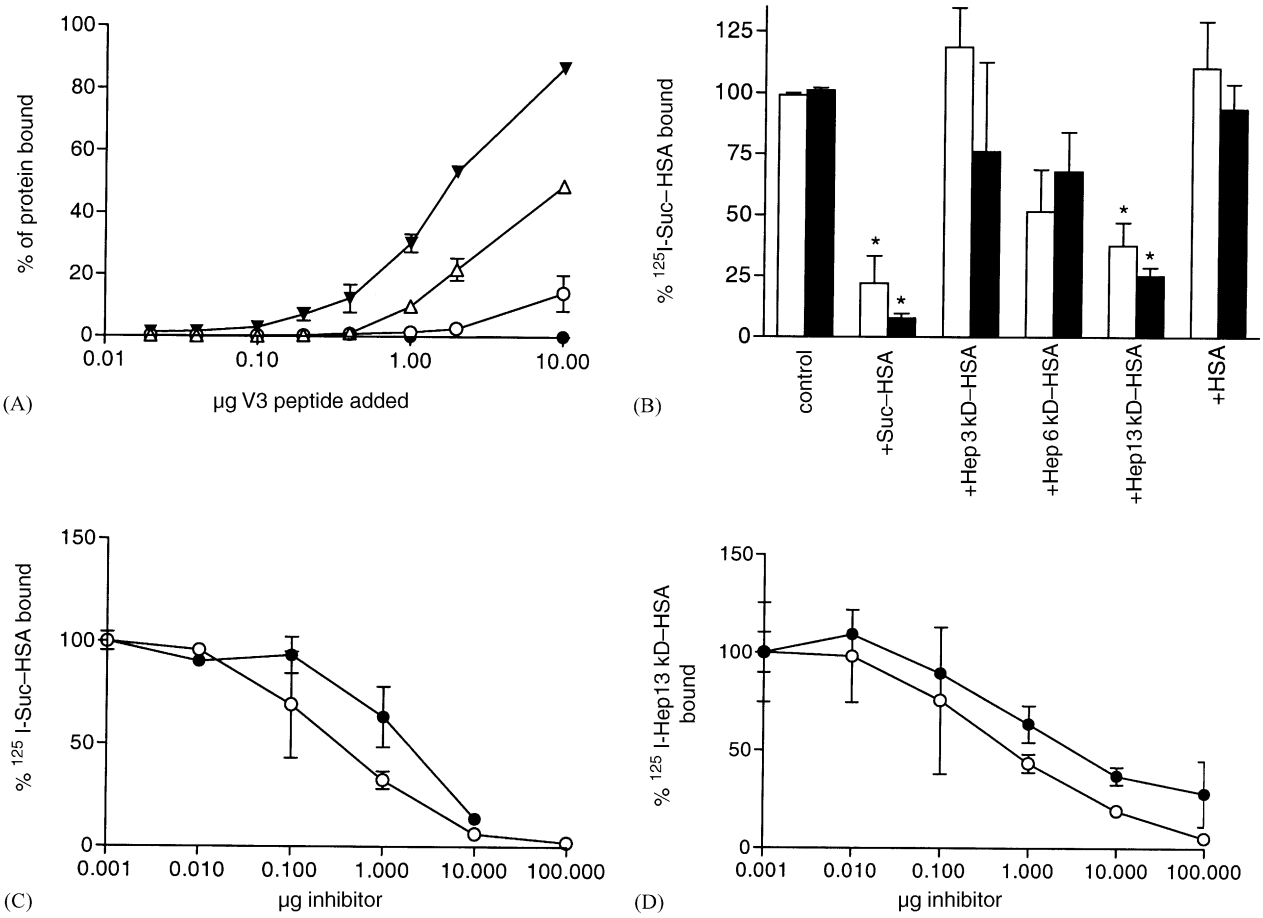
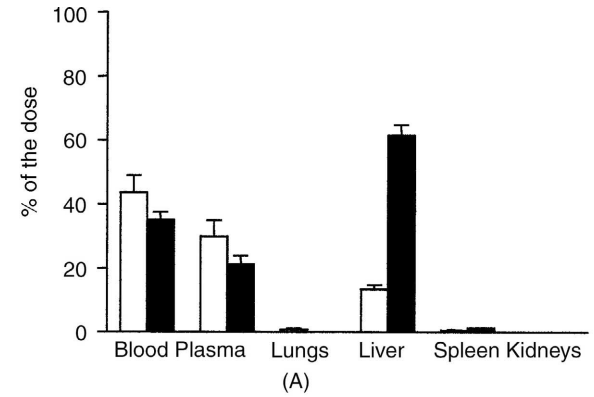


Fig. 3. Binding of the negatively charged albumins to the V3 loop of gp120. (A) Direct binding of ^{125}I-labeled Suc–HSA (–▼–), Hep3 kD–HSA (–○–), Hep13 kD–HSA (–△–) and HSA (–●–) to linear V3Q17 peptides. (B) Inhibition of ^{125}I-Suc–HSA binding to V3Q17 peptide by 20 μg/mL (white bars) or 200 μg/mL (black bars) modified albumin. (C) Inhibition of ^{125}I-Suc–HSA binding by increasing concentrations of Suc–HSA (○) and Hep13 kD–HSA (●). (D) Inhibition of ^{125}I-Hep13 kD–HSA binding by increasing concentrations of Hep13 kD–HSA (●) and Suc–HSA (○). Means ± SD (N = 3).



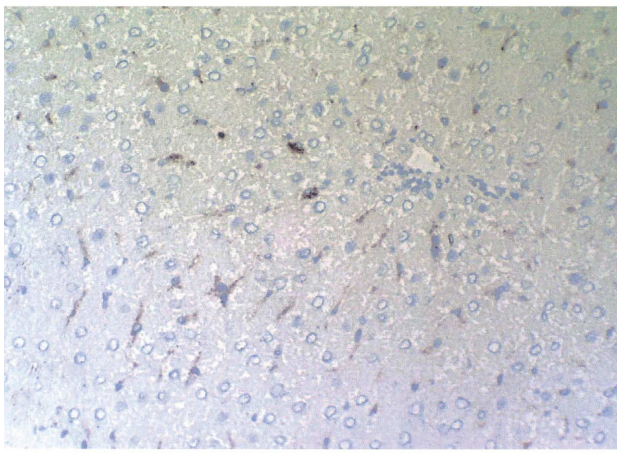


Fig. 4. *In vivo* behavior of heparin modified albumins after intravenous administration. (A) Organ distribution of ^{125}I-labeled Hep3 kD–HSA (open bars) and Hep13 kD–HSA (closed bars) at 10 min after injection of a tracer dose. (B) Immunohistochemical localization of Hep13 kD–HSA ($D = 4$ mg/kg) in the liver at 10 min after injection. Other organs did not stain positive for the modified albumin.

3.5. Pharmacokinetic behavior of Hep–HSA in vivo

3.5.1. Organ distribution

Ten minutes after i.v. injection of the heparin modified albumins to rats, the proteins were mainly present in blood and the liver (Fig. 4A). HSA-modified with Hep13 kD showed a more rapid uptake in the liver as compared to Hep3 kD–HSA. The hepatic contents were respectively 60.2 ± 4.0 and $19.8 \pm 0.5\%$ of the dose, whereas respectively 35.1 ± 2.5 and $43.7 \pm 5.3\%$ of the dose was still present in blood. No uptake was found in the spleen, kidneys or other organs. Using immunohistochemical analysis,

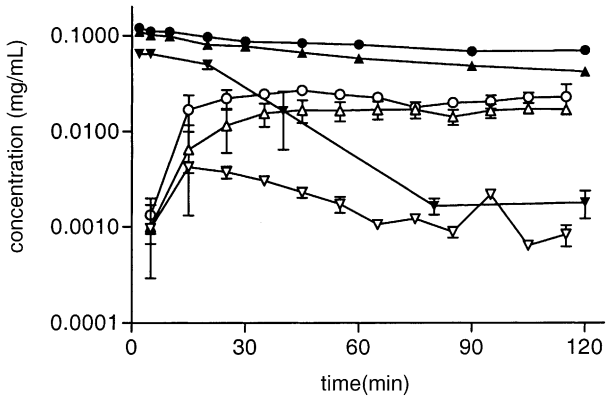


Fig. 5. Plasma (closed symbols) and lymphatic (open symbols) concentrations 2 hr after injection of 4 mg/kg ^{125}I-labeled Hep3 kD–HSA (–○–), Hep13 kD–HSA (–△–), and Suc–HSA (–▽–). Means ± SD (N = 3).

Hep13 kD–HSA^{-I} was detectable in the liver at 10 min after i.v. administration. The staining pattern corresponded with a non-parenchymal cell distribution (Fig. 4B). In the other organs, i.e. kidneys, lungs and spleen, no staining for the modified albumin was detected.

3.5.2. Plasma and lymphatic concentrations

The plasma concentrations of the Hep–HSA preparations (^{125}I labeled) slowly decreased in time as compared to a similar dose (4 mg/kg) of the randomly modified Suc–HSA (Fig. 5). Both the Hep–HSA and the Suc–HSA plasma curves followed 1-compartment kinetics after i.v. injection of 4 mg/kg, with kinetic parameters summarized in Table 4. The less rapid clearance of Hep–HSA from the plasma as compared to Suc–HSA was reflected by lower Cl and higher plasma $t_{1/2}$ values. This was also reflected by the mean residence time (MRT) of the compounds in plasma, which were significantly higher after 4 mg/kg Hep–HSA as compared to Suc–HSA. The volume of distribution was comparable for all compounds studied. It can also be derived from Table 4 that the coupling of a hydrophobic molecule to Hep–HSA, such as FiTC, did not significantly alter its pharmacokinetic properties.

Both Hep–HSA preparations accumulated in the lymphatic system (Fig. 5). As compared to Suc–HSA, more prolonged and higher concentrations were obtained in the lymph fluid. The patterns roughly followed the plasma-concentration profiles. The measured radioactivity consisted of TCA-precipitable material, indicating (active)

Table 4
Pharmacokinetic parameters of Hep13 kD–HSA, Hep3 kD–HSA, and Suc–HSA after fitting of the plasma curves as can be seen in Fig. 5

	$t_{1/2}$ (min)	Cl (mL/min)	V_1 (mL)	MRT (min)
^{125}I-Hep3 kD–HSA	162 ± 30	0.040 ± 0.007	9.4 ± 0.9	234 ± 43
^{125}I-Hep13 kD–HSA	86 ± 6	0.079 ± 0.006	9.8 ± 0.4	124 ± 8
FTC–Hep3 kD–HSA	367 ± 105	0.024 ± 0.007	12.5 ± 0.7	529 ± 151
FTC–Hep13 kD–HSA	97 ± 14	0.076 ± 0.044	13.6 ± 6.2	139 ± 20
^{125}I-Suc–HSA	14 ± 3	0.487 ± 0.092	9.5 ± 2.2	20 ± 4

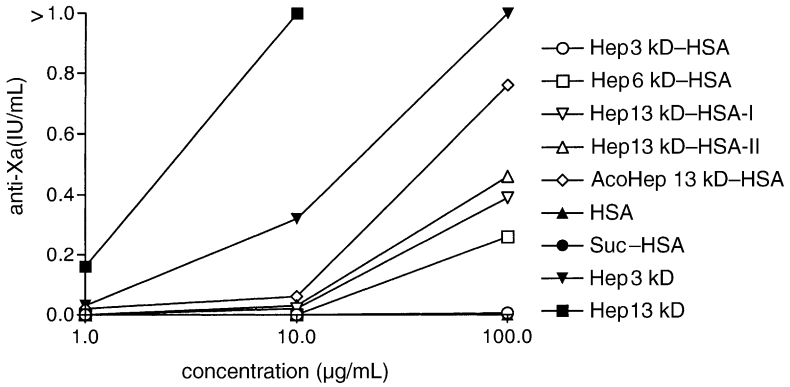


Fig. 6. Anti-coagulant activities of the various negatively charged albumins as measured with the anti-Xa assay. Heparin 3 and 13 kD were used as positive controls, HSA as a negative control.

transport of intact protein from the systemic circulation into the lymphatics. The concentrations of Hep13 kD–HSA attained in the lymph fluid approximated the *in vitro* IC_{50} concentration of its anti-HIV activity (i.e. 20 μg/mL, see Table 3). Accumulation in lymph was induced by the negative charge on HSA as HSA itself hardly accumulated in this compartment after i.v. injection (data not shown).

3.6. Anticoagulant activity of negatively charged proteins

Because anticoagulant activities of heparin are not desirable in case of application of Hep–HSAs for anti-HIV treatments, we determined whether the heparin modified albumins interfered with blood coagulation. The APTT test indicated that the coagulation times of Hep13 kD–HSA were normal (normal coagulation time = 27–33 s) for 1 and 10 μg/mL, concentrations at which uncoupled heparin 13 kD already delayed the blood coagulation (respectively 58 and >200 s). Only at the highest concentration studied (100 μg/mL), Hep13 kD–HSA prolonged the coagulation time although to a lesser extent than uncoupled Hep13 kD. Since the APTT assay is not appropriate to determine interference of the lower molecular weight heparins with blood coagulation, we also used an anti-Xa assay (Fig. 6). Also, higher anti-Xa values were measured only at 100 μg/mL concentrations for the small molecular weight heparins coupled to HSA. The values were enhanced with increasing chain length of heparin, but were never as high as the uncoupled heparins. The negatively charged albumin Suc–HSA and HSA itself did not interfere with blood coagulation up to 100 μg/mL in both assays. However, at *in vitro* anti-HIV IC_{50} values (5–20 μg/mL), no anticoagulation activity of Hep13 kD–HSA was found.

4. Discussion

Negatively charged albumins are potent inhibitors of HIV infection [7,8]. In this study we investigated whether the distribution of the charges on the albumin backbone influenced to the anti-HIV activity. For this, heparin fragments were covalently coupled to HSA. The globular conformation and structure of the heparinized albumins were compared to the acylated albumins. To determine the relation between structure and activity, we studied the *in vitro* anti-HIV activity (IC_{50}) and V3 loop binding potential. Furthermore, *in vivo* behavior of these compounds was investigated, since the kinetic profile of the compounds is an important factor that will affect antiviral effectiveness and therapeutic potential *in vivo*.

Chemical modification of proteins may affect the overall protein structure and hence influence cellular binding and *in vivo* kinetics. Therefore the effect of modification with heparin (3 and 13 kD) was studied using ^{1}H-NMR and DSC. Both analyses revealed that the globular structure of HSA was hardly affected upon modification with heparin. However, succinylation led to unfolding of the protein molecule as can be seen from comparing the ^{1}H-NMR spectra of Suc–HSA with that of HSA in 8 M urea and the lack of an endothermic energy change in the DSC experiments.

DLS experiments show that at neutral pH the covalently attached heparin polymers are folded onto the surface of the protein molecule, rather than protruding into the solution. This result seems logical considering the low pI (around 5.4) of HSA and the highly negatively charge of heparin at neutral pH. From this it is concluded that the heparin-modified molecules have a globular protein structure with a 'layer' of highly charged heparin polymers. In contrast, Suc–HSA is a highly negatively charged randomly coiled protein structure.

HSA substituted with 13 kD heparin displayed the strongest anti-HIV activity. This activity can, at least partly, be explained by binding to the V3 loop of gp120. As previously shown for the acylated albumins, this shielding of gp120 can largely interfere with the cellular binding of HIV particles with its target cells [9]. This molecular interaction was also described for heparin itself [4,27]. As far as the composition of heparin is concerned, Rider *et al.* [27] found that decreasing the number of negative charges by selective O- or N-desulfation of heparin, resulted in reduced anti-HIV activity. The observation that

Hep13 kD–HSA was able to inhibit the binding of Suc–HSA to the V3 loop, indicated either that both compounds interacted with the same epitope in the V3 region, i.e. GPGRAF [10], or that a competition between the two modified albumins occurred due to steric hindrance while binding to another (close) epitope. The SI variant of the V3 peptide (V3-Q17) displayed much stronger interactions with the negatively charged albumins as compared to the NSI peptide, which may be explained by the more cationic character of the SI peptide. In addition, it might be interesting to compare *in vitro* antiviral activities of the negatively charged proteins using NSI and SI strains of HIV. This was not assessed with Suc–HSA and Hep–HSA. However, another negatively charged protein, 3-hydroxyphtalic acid betalactoglobulin (3-HP-bLG), did not show a preference, that is it inhibited various SI and NSI strains to a similar extent (Berkhout *et al.*, personal communication).

The randomly modified (acylated) albumins exhibited stronger anti-HIV activities *in vitro* compared to the clustered variants (Hep–HSA preparations), in spite of their similar overall charge modification (all proteins have pI 2.4). These data correlated with the observed better interaction of Suc–HSA with the V3 loop. Furthermore, co-substitution of aconitic acid groups and heparin 13 kD to HSA resulted in significantly lower IC_{50} values as compared to Hep13 kD–HSA. Thus, in addition to the previously ascertained structural prerequisites, i.e. the charge density on a protein backbone and an intact globular structure [8], we can now conclude that an overall distribution of negative charges on the albumin molecule is preferred for optimal anti-HIV activity. Neurath *et al.* [15] demonstrated that milk proteins (albumin or casein) modified with aromatic anhydrides, such as 3-hydroxyphtalic acid, were more effective as compared to aliphatic anhydrides, implicating that in addition to negative charge, hydrophobic interactions also play a role in anti-HIV activity.

In vivo studies demonstrated that Hep–HSA was present in the blood for a prolonged time, and distributed rapidly to the lymphatic system, as did the succinylated HSA [26]. Yet, Hep–HSA was less efficiently cleared by the liver as reflected by a higher plasma $t_{1/2}$ and may therefore perform a more prolonged interaction with HIV particles in the body. The higher concentrations of Hep–HSA compared to Suc–HSA obtained in blood and lymph, two important compartments with regard to the dissemination and residence of HIV [28], may imply that better anti-HIV activity may be obtained in the *in vivo* situation. However, this aspect is dose dependent since higher doses Suc–HSA or Aco–HSA also have long residence times in the blood stream, due to saturation of scavenger receptor modified clearances [12,26].

The negatively charged albumins investigated in this study all possess anti-HIV activity. Due to the *in vivo* preference of Suc–HSA for blood cells, these proteins may also be used as a carrier molecule to deliver antiviral drugs, like zidovudine (AZT), to HIV prone cells [29]. The uptake of AZT in non-target tissue will be limited that case, because the cellular uptake of AZT is now determined by the *in vivo* distribution of the negatively charged albumins, and not anymore by the characteristics of AZT. After release of AZT from this negatively charged protein inside the target cells, the reverse transcriptase inhibitor can exert a cell-selective effect. Moreover, synergistic effects may then be expected because of a simultaneous inhibition of HIV replication at the intracellular and the membrane levels [30]. In contrast to Suc–HSA, the Hep–HSA preparations were not taken up in spleen, kidneys and lungs. This may be important with regard to potential side-effects of the modified protein or the coupled drugs at those sites.

In conclusion, Hep13 kD–HSA, with clustered negative charges, showed anti-HIV activity, likely due to binding to gp120 V3 loop. In contrast, random modification of HSA with negatively charged groups, as obtained by acylation of HSA (Suc–HSA and Aco–HSA), resulted in more potent anti-HIV activity *in vitro*. Yet, *in vivo* studies showed longer plasma $t_{1/2}$ and higher lymphatic concentrations (both important HIV reservoirs) for Hep–HSA in the given doses. These kinetic features combined with its lack of anticoagulant activity make Hep13 kD–HSA an attractive anti-HIV protein for further study in spite of the less potent anti-HIV activity. A real antiviral activity of the charged compounds that interfere with the initial phase of the virus life cycle can only be properly assessed *in vivo*. A few of entry/fusion inhibitors are in clinical trials now, including the peptide inhibitor T20 and the CXCR4 antagonist bicyclam AMD3100 [31].

Acknowledgments

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