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Pharmacokinetics and anti-HIV-1 efficacy of negatively charged human serum albumins in mice

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Abstract

Negatively charged albumins (NCAs, with the prototypes succinylated human serum albumin (Suc-HSA) and aconitylated human serum albumin (Aco-HSA)), modified proteins with a potent anti-human immunodeficiency virus type 1 (anti-HIV-1) activity in vitro, were studied for their pharmacokinetic behaviour in mice and their in vivo anti-HIV-1 efficacy in an HIV-1 infection model in mice. In contrast to the saturation kinetics found in rats, intravenous injections of 10–300 mg/kg for both NCAs showed a linear correlation between the area under the curve (AUC) and the dose. The elimination $t_{1/2}$ was 25 and 30 min for Suc-HSA and Aco-HSA, respectively. Preinjections of an excess of formaldehyde-treated albumin (Form-HSA) resulted in plasma levels that were 3- and 4-fold higher for Aco-HSA and Suc-HSA, respectively. These data indicate that elimination is at least partly (scavenger) receptor-mediated. Organ distribution studies 10 min after injection showed an accumulation in liver (Suc-HSA $17.3 \pm 6.6\%$ of the dose; Aco-HSA $20.9 \pm 2.3\%$) and lungs (Suc-HSA $12.7 \pm 10.5\%$; Aco-HSA 16.0 ± 13.6). Intraperitoneal injection of 300 mg/kg Suc-HSA resulted in a final bioavailability of about 0.45. Suc-HSA was also evaluated for its in vivo neutralizing capacity in a human-to-mouse chimeric model for HIV-1 infection. Intraperitoneal injections of 300 and 3 mg/kg Suc-HSA, given 15–30 min before the mice were challenged with the virus, sufficed to protect these mice against infection with the HIV-1 III_B strain. Copyright © 1997 Elsevier Science B.V.

Keywords: Human immunodeficiency virus type 1; Negatively charged human serum albumins; Antiviral; Polyanion

Abbreviations: Aco-HSA, Aconitylated human serum albumin; GvHD, Graft versus host disease; ICT, Infectious centre test; NCA, Negatively charged human serum albumin ; PBMC, Peripheral blood mononuclear cells; Suc-HSA, Succinylated human serum albumin.

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1. Introduction

Negatively charged human serum albumins (NCAs) are very potent in vitro anti-human immunodeficiency virus type 1 (anti-HIV-1) compounds, as we described previously (Jansen et al., 1991b, 1993b; Swart and Meijer, 1994). The modification of albumin by either succinylation or aconitylation, by which extra anionic groups are introduced into the protein, resulted in a new class of anti-HIV-1 compounds with the prototypes succinylated human serum albumin (Suc-HSA) and aconitylated human serum albumin (Aco-HSA), respectively. These charged modified proteins showed a profound in vitro anti-HIV-1 activity (50% inhibitory concentration (IC_{50}): Suc-HSA 0.2 μg/ml; Aco-HSA 0.03 μg/ml) and act at the level of virus–cell fusion (Jansen et al., 1991a,b, 1993a,b; Swart and Meijer, 1994; De Clercq, 1995). Recently, the binding of the NCAs to the V3 loop of the envelope glycoprotein gp120 of HIV-1 was reported (Kuipers et al., 1996). From these experiments it was suggested that the mechanism of anti-HIV-1 activity of the NCAs may therefore (at least partially) be explained by this V3 loop binding.

However, promising in vitro data do not guarantee in vivo anti-HIV-1 efficacy, as was seen earlier for dextran sulfate and rCD4 (Hartman et al., 1990; Yarchoan et al., 1990; Flexner et al., 1991). An adequate pharmacokinetic profile as well as a proper biocompatibility would certainly represent other requirements for an antiviral therapy in vivo. Pharmacokinetic screening of Suc-HSA and Aco-HSA in rats earlier indicated that in vivo anti-HIV-1 activity is in principle possible (Jansen et al., 1993a; Swart et al., 1996a): at doses of least 15 mg/kg, a saturable elimination process could be observed, with plasma concentrations of the NCAs far exceeding their in vitro anti-HIV-1 IC_{50} values and persisting for more than 6 h after a single dose. No acute toxicity (400 mg/kg once) or sub-acute toxicity (40 mg/kg once a day for 2 weeks) were observed in rats (Swart and Meijer, 1994).

In the present study, the pharmacokinetic behaviour of Suc-HSA and Aco-HSA in mice is analyzed. We studied the plasma disappearance and organ distribution of both compounds after intravenous (i.v.) and intraperitoneal (i.p.) injections. This pharmacokinetic screening enabled us to establish a dosage regimen by which a significant antiviral activity in mice should be expected. Subsequently, the efficacy of Suc-HSA was investigated in a human-to-mouse chimeric model, the xeno-GvHD model (Huppes et al., 1992). In this model, immunosuppressed CBA/N mice are grafted with large numbers of human peripheral blood mononuclear cells (PBMC), resulting in an acute graft versus host disease (GvHD) reaction, with highly activated immunocompetent cells. The kinetics of HIV-1-III_B infection and the antiviral activity of HIV-1-III_B-specific antibody preparations in this mouse model have recently been described (Schutten et al., 1996). HIV-1-III_B challenge was performed approximately 1 h after grafting of the human PBMC and long before the characteristic activation of the human PBMC graft takes place. Another difference between this model and the previously described human-to-mouse chimeric models (McCune et al., 1990; Mosier et al., 1991) is the presence of large numbers of human macrophages in the peritoneal cavity. In primary HIV-1 infection in humans, macrophages are the primary target cells (Gartner and Popovic, 1990). In the present mouse model, Suc-HSA was tested for its ability to protect the human cells in these mice against the HIV-1 virus. For this reason it was also given i.p.. Suc-HSA was injected 15–30 min before the mice were challenged with HIV-1, to obtain a maximal protective effect locally. In addition, as a result of diffusion of Suc-HSA from the peritoneal space into the general circulation, a sustained release of the antiviral protein in the blood stream occurred, leading to plasma concentrations that may be sufficient to inhibit viral replication also in other parts of the body.

2. Materials and methods

2.1. Synthesis and characterization of NCAs

Derivatization of HSA (Central Laboratory of the Netherlands Red Cross Blood Transfusion Services, Amsterdam, The Netherlands) with succinic anhydride or *cis*-aconitic anhydride was per-

formed as previously described (Jansen et al., 1991b, 1993b). Protein concentration was determined according to Lowry et al. (1951). Determination of the free ε-NH_2 group of the lysines of the derivatized HSAs was performed according to Habeeb (1966). The percentage monomeric conjugate as well as the relative net negative charge of the modified albumins was determined according to Kuipers et al. (1996).

2.2. Radioiodination

Proteins were labelled with ^{125}I to a specific activity of 5 Ci/g using a chloramine-T method (Greenwood et al., 1963). Unattached ^{125}I was either removed by gel filtration on a Sephadex G25 column or by dialysis prior to experiments. Radioactivity used in the experiments was more than 95% precipitable with trichloroacetic acid (TCA, 20%). Radioactivity was counted in a LKB Multichannel gamma counter (LKB, Bromma, Sweden).

2.3. In vivo pharmacokinetic behaviour of NCAs

2.3.1. Plasma clearance

Male Swiss mice (HsD:NIHS, outbred strain, Harlan, Zeist, The Netherlands) (15–25 g) were anaesthetized with pentobarbital (Nembutal, 60 mg/kg i.p.). NCAs spiked with ^{125}I-labelled protein (1.5×10^5 cpm/100 μl) were injected into tail vein. At indicated time points (2–60 min), blood samples of 75 μl were taken from the tail vein from each mouse using heparinized blood capillaries and centrifuged at $5600 \times g$ for 5 min to obtain plasma. Plasma samples were mixed with 200 μl TCA (20%) and centrifuged at $5600 \times g$ for 5 min to differentiate between large molecules and small peptides as described earlier by Jansen et al. (1993a). Radioactivity of the supernatant and the pellet was counted as described above.

2.3.2. Organ distribution

A tracer amount of ^{125}I-labelled NCA (1.5×10^5 cpm/100 μl) was injected as described above. After 10 min, the mice were killed and the various organs were removed and washed with a physiological salt solution before radioactivity was counted as described above. The effect of a scavenger receptor blocker was also studied; for this, 150 mg/kg formaldehyde-treated HSA (Form-HSA) (Jansen et al., 1991a) was injected into the tail vein 5 min prior to the administration of the iodinated NCA.

2.3.3. Plasma concentrations after i.p. injection

Mice were injected i.p. with ^{125}I-labelled NCAs (300 mg/kg) as described above. At indicated time points (0–6 h), blood samples were taken from the tail vein and various organs were removed.

2.4. Data analysis

Plasma concentration–time curves were analyzed with the curve fitting program MW/PHARM (Mediware, Groningen, The Netherlands) (Proost and Meijer, 1992). The apparent parameters AUC (area under curve), volume of distribution (V_{ss}) and the elimination half-life time ($t_{1/2}$) were calculated using single-compartment analysis. Bioavailability after i.p. injection was assessed with the help of a numerical deconvolution technique, using the fitting program KinBes (Mediware, Groningen, The Netherlands), with the pharmacokinetic parameters obtained after i.v. injection of a comparable dose as a reference (Proost and Meijer, 1992; Proost, 1985, 1987).

2.5. Anti-HIV-1 efficacy of NCAs

The xeno-GvHD mouse model is used as described in detail previously by Huppes et al. (1992). For HIV-1 infection in the xeno-GvHD mouse model, the method recently described by Schutten et al. (1996) was used. In short, male immunosuppressed CBA/N/Rij mice (Harlan, 4–5 weeks old, IgM^-) were conditioned 1 day before the start of the experiments by total body irradiation (9 Gy) with haematological support of 5×10^5 syngeneic bone marrow intravenously. Human PBMC used for grafting were isolated using Ficoll gradient from blood taken from healthy blood donors at the Blood Bank, Rotterdam. In the experiments 2×10^7 PBMC/g body

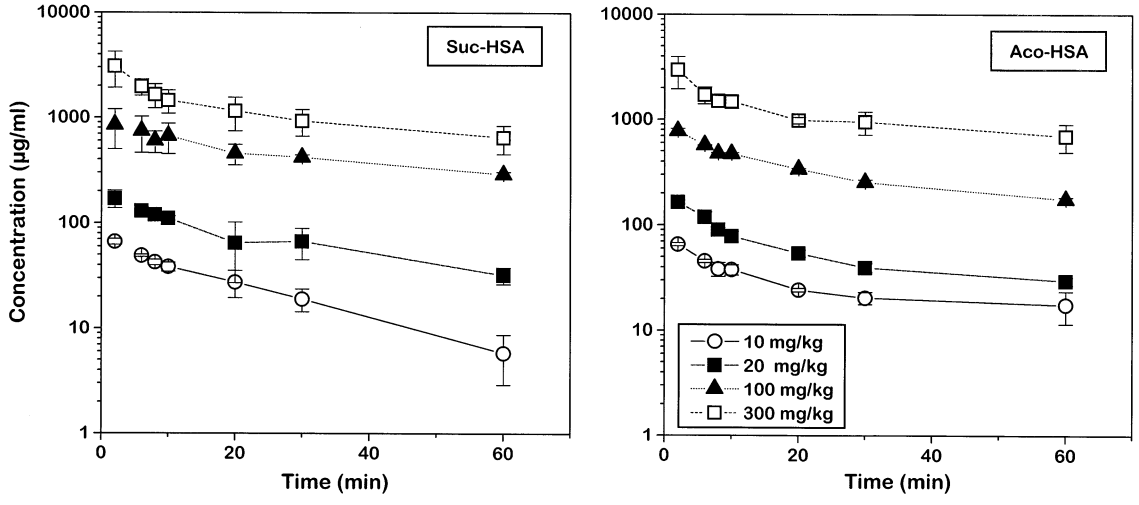

Fig. 1. Plasma concentration–time profiles of Suc-HSA (left) and Aco-HSA (right) following i.v. injections of 10 mg/kg ($n = 3$), 20 mg/kg ($n = 2$), 100 mg/kg ($n = 2$) and 300 mg/kg ($n = 4$) in mice. The results represent acid-precipitable radioactivity ($\pm$ S.E.M.).

weight were given i.p., which have been shown to induce an acute GvHD in almost 100% of the cases (Huppes et al., 1992). Suc-HSA was given in three different dosages of 0.3, 3 and 300 mg/kg i.p. 15–30 min before the mice were challenged with HIV-1 (III_B-strain). When GvHD was observed in these mice at day 6 (death of at least one mouse in the group with acute xeno-GvHD symptoms, i.e., wasting, furry coat, breathing problems and internal bleedings), the human cells from the peritoneal cavity were isolated. An infectious centre test (ICT) was used to determine the viral load of the human lymphocytes isolated from the peritoneal washing. The human lymphocytes from the peritoneal lavages were counted and titrated in duplicate starting at 2.5×10^6 per well in 96-well round bottom plates. The cells were cultured in the presence of phytohaemagglutinin (PHA)-prestimulated human PBMCs (5×10^4 per well). After 1 week, the cells were tested for the presence of p24 antigen using an enzyme-linked immunosorbent assay (ELISA) (V5 p24 antigen ELISA kit, a kind gift of Organon Teknika, The Netherlands). The amount of cells from the peritoneal lavage needed to detect viral antigen in more than 50% of the wells was taken as a measure for the viral load.

3. Results

3.1. In vivo pharmacokinetic behaviour of NCAs

Dosages of 10–300 mg/kg of Suc-HSA or Aco-HSA were injected i.v. in mice and the resulting plasma concentration–time profiles are shown in Fig. 1. For both compounds, a linear correlation between the injected dose and the AUC was observed, which indicates first-order pharmacokinetic conditions.

Pharmacokinetic analysis, using the curve fitting program MW/PHARM (Proost and Meijer, 1992), revealed comparable kinetic data for both NCAs, as is summarized in Table 1. Using single-compartment analysis, the clearance (derived by the calculated AUC), the volume of distribution and the elimination half-life time were calculated. The results obtained in Swiss mice did not differ from pharmacokinetic data obtained for the NCAs in CBA/N mice (results not shown).

The organ distribution (Fig. 2) at 10 min after injection of tracer amounts of ^{125}I-labelled Suc-HSA or ^{125}I-labelled Aco-HSA in mice showed an accumulation in liver (Suc-HSA $17.3 \pm 6.6\%$ of the dose; Aco-HSA $20.9 \pm 2.3\%$) and lungs (Suc-HSA $12.7 \pm 10.5\%$; Aco-HSA $16.0 \pm 13.6\%$).

Table 1
Pharmacokinetic parameters of Suc-HSA and Aco-HSA obtained after i.v. injection of 10–300 mg/kg in mice, as described in Section 2

Compound	Cl_m (ml/min per kg)	V_{ss} (ml/kg)	$t_{1/2}$ (min)	Bioavailability after i.p. injection	Lag-time[a] (min after i.p. injection)
Suc-HSA	4.0 ± 0.3	142 ± 9.8	24.6	0.45	13.4
Aco-HSA	3.9 ± 0.2	172 ± 8.0	30.6	N.d.	N.d.

N.d., not determined.
[a] Time between i.p. injection and first appearance in blood.

Preinjections with Form-HSA increased the plasma concentration at 10 min about 3- and 4-fold for Aco-HSA and Suc-HSA, respectively. The total amounts in liver and spleen, however, were not reduced by pre-administration of Form-HSA (150 mg/kg). Organ distribution after 1 h showed comparable distribution patterns for both NCAs (mainly liver and lungs), although a certain amount (5–10%) of radioactivity was located in the kidneys after 1 h, probably due to the presumed low-molecular-weight degradation products of the labelled proteins (results not shown).

I.p. injections of 300 mg/kg Suc-HSA (Fig. 3) showed a rapid absorption into the general circulation (lag time: 13.4 min). The obtained plasma levels with this dosage were far above the in vitro IC_{50} values of Suc-HSA (300 μg/ml versus 0.2 μg/ml). After 6 h, the bioavailability following i.p. injection, calculated from the plasma decay curves after i.v. and i.p. administration of 300 mg/kg (as described in Section 2) was 0.45.

3.2. Anti-HIV-1 efficacy of NCAs

The results of Suc-HSA in the xeno-GvHD mice challenged with HIV-1 (III_B strain) are shown in Fig. 4. I.p. injections of 3 and 300 mg/kg Suc-HSA 15–30 min before challenge with the virus sufficed to completely protect the human grafts from HIV-III_B infection, whereas an i.p. injection of 0.3 mg/kg only partially protected the grafts.

4. Discussion

In this study, we demonstrated for the first time the anti-HIV-1 efficacy of negatively charged human serum albumins in an in vivo human-to-mouse chimeric model for HIV-1 infection. A clear dose–response relation could be determined for Suc-HSA against the HIV-1 III_B strain in the xeno-GvHD mouse model.

Preceding pharmacokinetic studies of the NCAs in mice enabled us to predict a dose regimen that in principle should also lead to inhibition of HIV-1 replication in vivo. Both NCAs demonstrated a similar linear pharmacokinetic behaviour in the present study in mice. Our results indicate that both NCAs should in principle be antivirally active when given at a dose of at least 10 mg/kg. At this dose, the plasma concentrations of Suc-HSA and Aco-HSA largely exceed the in vitro IC_{50} values for at least 4–6 h, if the pharmacokinetic parameters depicted in Table 1 are used. For example, the plasma concentration of Suc-HSA after an i.v. injection of 10 mg/kg per mouse after 4 h is calculated at 0.18 μg/ml, whereas the antiviral IC_{50} value determined in vitro is 0.2 μg/ml. The elimination $t_{1/2}$ value obtained in the short term (1 h) study is well in line with the results obtained in the extended (6 h) experiment (Fig. 3). On the basis of this kinetic profile, even the dose of 0.3 mg/kg NCAs (the lowest dosage used in the infection model) predicts plasma concentrations of Suc-HSA and Aco-HSA exceeding the in vitro IC_{50} values for 2–4 h after injection.

The distribution volume of the NCAs in the mice was 4-fold higher than the theoretical plasma volume. Either some accumulation in blood cells occurred or some of the modified proteins may have left the general circulation. In a preliminary study, we detected presence of NCAs in lymph fluid and passage of the drug through the endothelium is therefore likely (Swart and Meijer,

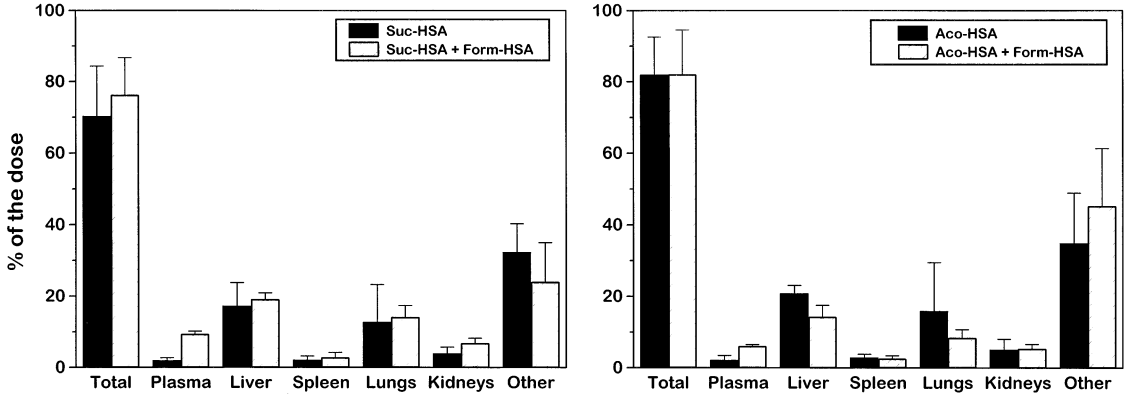

Fig. 2. Tissue distribution of a tracer amount of Suc-HSA (left) and Aco-HSA (right) 10 min after i.v. injection in mice with or without preinjection of Form-HSA ($n = 3 \pm$ S.E.M.).

1994). At the same time, at least some binding to macrophages and T-lymphocytes via various scavenger receptors is likely (Takami et al., 1992; Kuipers, unpublished results).

I.p. injections lead to a rapid absorption from the peritoneal space into the general circulation. The final bioavailability of Suc-HSA ($t = 0$ to $t = \infty$) was 0.45. We assume that i.p.-administered Suc-HSA reached the general circulation via uptake by the lymphatic system. As mentioned above, exchange between the lymphatic and blood compartments was demonstrated in our laboratory for these types of compounds (Swart and Meijer, 1994). It is unlikely that a substantial quantity of the i.p.-injected Suc-HSA is removed by first pass through the liver. High hepatic extraction is not to be expected since the hepatic clearance of NCAs is relatively low compared with the hepatic blood flow. Furthermore, the pattern of tissue accumulation of Suc-HSA in various organs after i.p. injections was very similar to that after i.v. injection. Therefore the remaining Suc-HSA in the peritoneal space is probably subject to metabolic degradation by enzymes abundantly present in the peritoneal fluid.

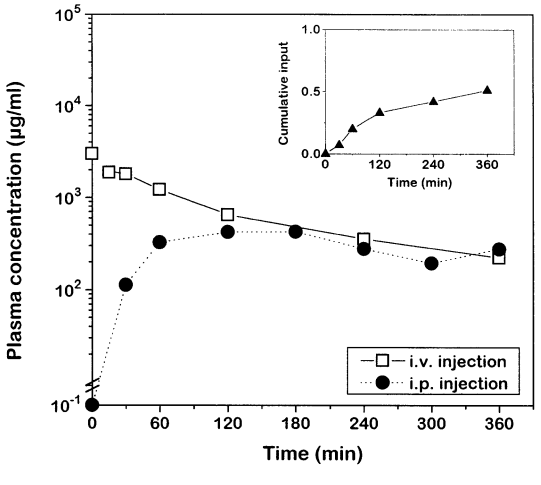

Fig. 3. Plasma concentration–time profile of Suc-HSA following i.v. or i.p. injection in mice. Inset: cumulative input of Suc-HSA in plasma after i.p. injection. The results represent acid-precipitable radioactivity ($n = 2$).

The elimination patterns of the NCAs observed in the present study were somewhat unexpected, because earlier studies with these compounds in both rats and monkeys (Jansen et al., 1993a; Swart et al., 1996a,b) showed a clear Michaelis–Menten type of elimination. Furthermore, in a study by Takakura et al. (1994), in which the pharmacokinetics of succinylated bovine serum albumin was studied in mice, the hepatic uptake at higher doses was relatively low, probably due to a saturable uptake process mediated via scavenger receptors. The use of a different mouse strain in the latter study as well as the use of a different modified albumin (not all available ε-NH_2 groups in the albumin were derivatized by succinylation) could

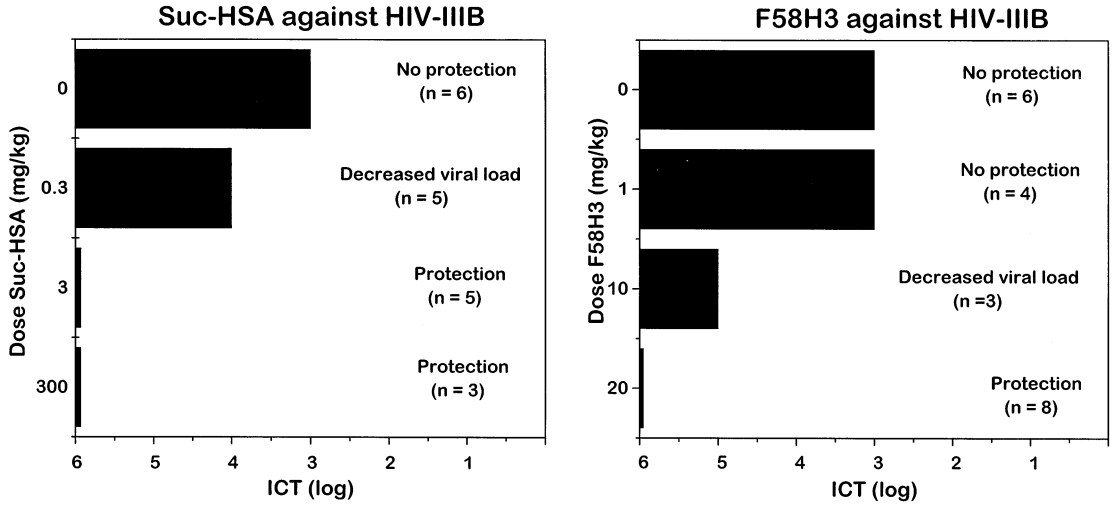

Fig. 4. Dose-efficacy relation of Suc-HSA in xeno-GvHD mice infected with HIV (III_B strain). Cell-associated virus load was determined by ICT in human cells from the peritoneal lavages of xeno-GvHD mice at day 6 after infection. The ICT is the amount of cells from the peritoneal washing needed to detect viral antigen in more than 50% of the wells (see Section 2). The horizontal axis represents the logarithm of the numbers of cells needed to yield positive cultures in the ICT. If more than 10^6 cells are indicated, none of the mouse tissues (peritoneal tissue, spleen, ascitic fluid cells) also tested in culture proved to be positive for HIV-1 p24 after 6 days. Left, Suc-HSA; right, V3-loop-specific mAb F58H3 (mouse IgG_1 antibody (Broliden et al., 1992)). Data taken from Schutten et al. (1996).

explain the differences in pharmacokinetics compared with our study. In the above-mentioned studies, the dose-dependent elimination pattern of the NCAs was thought to be caused by saturation of the hepatic scavenger receptors. The avid liver uptake of the NCAs (up to about 70–80% of the dose, in these studies) was reported to be reduced by preinjections of ligands for the scavenger receptor (Form-HSA, poly-inosinic acid). However in our study we observed a maximal liver content of only 15–20% of the injected dose. Although preinjection of Form-HSA did not lower the absolute liver content of the NCAs compared with controls, the liver to plasma concentration ratio was 3- and 4-fold lower than the controls (for Aco-HSA and Suc-HSA, respectively). This indicates that the liver uptake process per se was at least partially inhibited by Form-HSA. However, lower tissue to plasma concentration values were also observed for the other tissues studied (see Fig. 2). From these results we conclude that scavenger receptor-mediated liver uptake of the NCAs in mice, in contrast to rats, is only contributing modestly to the total removal of the NCAs from the blood stream. Either there is a low density of functional scavenger receptors in the liver in these mice or the NCAs have a relatively low affinity for the hepatic scavenger receptors in particular. Nevertheless, the clear effect of a scavenger receptor ligand on the plasma clearance of the NCAs suggests the involvement of some sort of scavenger-mediated elimination pathway in various other tissues in addition to liver and spleen. This difference in elimination pattern between the rat and the mouse was further illustrated by the phenomenon that after i.p. injection in mice of 300 mg/kg Suc-HSA, a plasma concentration was observed that largely exceeds in vitro IC_{50} values, whereas in rats such concentrations were not attained at this dose, most likely because of the rapid clearance by the hepatic scavenger receptor system (Swart et al., 1996b).

On the basis of the present pharmacokinetic data in mice, it was calculated that in the xeno-GvHD mice, in which Suc-HSA was injected i.p.

in a dose range of 0.3–300 mg/kg, concentrations of Suc-HSA exceeding the in vitro anti-HIV-1 IC_{50} value are attained, both in the peritoneal space and in the plasma. For instance, 60 min after i.p. injection of 300 mg/kg Suc-HSA, a concentration of Suc-HSA of at least 300 μg/ml in plasma was detected, as is illustrated in Fig. 3. Extrapolated for a dosage of 0.3 mg/kg, this would result in plasma concentrations of Suc-HSA of 0.3 μg/ml, in the range of the in vitro IC_{50} of Suc-HSA.

We observed a complete protection against the HIV-1 III_B strain in the xeno-GvHD mice, using 3 or 300 mg/kg Suc-HSA. The concentration of Suc-HSA needed to neutralize HIV-1 III_B in the xeno-GvHD mouse system was approximately similar on a molar basis to that of the V3-loop-specific monoclonal antibody F58H3, as was reported by Schutten et al. (1996) recently (see also Fig. 4). In the present study, we detected no complete protection after an i.p. dosage of 0.3 mg/kg Suc-HSA. Since primary HIV-1 infection takes place in the peritoneal cavity (Schutten et al., 1996), at the site where the human PBMCs and HIV-1 particles are injected, a dosage of 0.3 mg/kg would theoretically be sufficient to largely prevent viral replication locally. In a recent study by Gauduin et al. (1995), however, it was suggested that for a proper in vivo inhibitory effect, the IC_{90} value is more relevant if complete protection is the required endpoint.

However, additional factors may explain the lack of complete effect at the lowest dose. Firstly, in a recent study by Swart et al. (1996c) it was shown that the in vitro antiviral activity of Suc-HSA is reduced about 10-fold when tested in blood plasma instead of in the commonly used culture medium. This phenomenon was reported for other antiviral compounds as well (Bilello et al., 1995; Hartman et al., 1990; Kageyama et al., 1994). Such a reduced antiviral efficacy of Suc-HSA may also have occurred in the peritoneal fluid. Secondly, some degradation of Suc-HSA may also occur in the peritoneal extracellular fluid and together with their lymphatic drainage may have lead to sub-protective levels for at least some time period. A third factor might be that some of the infected human cells migrated to the general circulation, in which, according to the present study, a maximum plasma concentration of only 0.3 μg/ml Suc-HSA can be expected. Taking into account the in vitro IC_{50} value, this plasma concentration is evidently too low to inhibit HIV-1 replication in the blood compartment over the entire period (Swart et al., 1996c).

In the present study, the antiviral compound was injected 15–30 min before the virus was injected. Consequently, Suc-HSA had the opportunity to immediately bind to the HIV-1 particles when injected into the mice. In earlier research in our laboratory, it was shown that the NCAs act early in the life cycle of the virus, namely on the fusion of the virus with the target cell (Swart and Meijer, 1994). This mechanism of action could partially be explained by binding of the NCAs to the V3 loop of gp120 (Kuipers et al., 1996), a domain of the envelope protein that is instrumental in the fusion process (Pinter et al., 1993; Bergeron et al., 1992). It would be interesting in the future to test the efficacy of the NCAs in vivo in time of addition experiments, since it is not excluded that the NCAs would be more efficient if the virus particles are first allowed to bind to the target cells. Such a phenomenon was recently observed by Yahi et al. (1995), who used a synthetic multibranched peptide that also inhibits the fusion of HIV-1 particles with the target cells. These authors demonstrated that preincubation of virus with the target cells increased the antiviral activity of this multibranched peptide when it was added at least 1 h after initial exposure of the target cells to HIV-1.

In conclusion, our results imply that NCAs are not only antivirally active in vitro but also in vivo, at least in the chosen infection model. We conclude therefore that these charged–modified proteins may be promising compounds to be tested in humans. Studies with other HIV strains in the mouse infection model and also in simian immunodeficiency virus (SIV)-infected monkeys are underway. Furthermore, commonly used anti-HIV-1 compounds, such as AZT, can be covalently coupled to these NCAs, as described by Molema and Meijer (1992) and, more recently, by Kuipers et al. (1995). The conjugates thus obtained (NCA-AZTMP) can be used as 'dual-

targeting' preparations, having inhibitory effects on both virus entry as well as on RNA/DNA transcription. Such combined activities potentially can lead to synergistic effects (Kuipers et al., manuscript in preparation) and could also limit the development of drug resistance in the on-going mutation and selection process during chronic therapy. The efficacy of these AZT conjugates of NCAs is presently being tested in the mouse model presented in this study.

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References

Bergeron, L., Sullivan, N. and Sodroski, J. (1992) Target cell-specific determinants of membrane fusion within the human immunodeficiency virus type 1 gp120 third variable region and gp41 amino terminus. J. Virol. 66, 2389–2397.

Bilello, J.A., Bilello, P.A., Prichard, M., Robins, T. and Drusano, G.L. (1995) Reduction of the in vitro activity of A77003, an inhibitor of human immunodeficiency virus protease, by human serum alpha 1 acid glycoprotein. J. Infect. Dis. 171, 546–551.

Broliden, P.A., von Gegerfelt, A., Clapham, P., Rosen, J., Fenyö, E.-M., Wahren, B. and Broliden, K. (1992) Identification of human neutralization-inducing regions of the human immunodeficiency virus type 1 envelope glycoproteins. Proc. Natl. Acad. Sci. USA 89, 461–465.

De Clercq, E. (1995) Antiviral therapy for human immunodeficiency virus infections. Clin. Microbiol. Rev. 8, 200–239.

Flexner, C., Barditch-Crovo, P.A., Kornhauser, D.M., Farzadegan, H., Nerhood, L.J., Chaisson, R.E., Bell, K.M., Lorentsen, K.J., Hendrix, C.W., Petty, B.G. and Lietman, P.S. (1991) Pharmacokinetics, toxicity, and activity of intravenous dextran sulfate in human immunodeficiency virus infection. Antimicrob. Agents Chemother. 35, 2544–2550.

Gartner, S. and Popovic, M. (1990) Macrophage tropism of HIV-1. AIDS Res. Hum. Retroviruses 6, 1017–1021.

Gauduin, M.-C., Safrit, J.T., Weir, R., Fung, M.S.C. and Koup, R.A. (1995) Pre- and post-exposure protection against human immunodeficiency virus type 1 infection mediated by a monoclonal antibody. J. Infect. Dis. 171, 1203–1209.

Greenwood, F.C., Hunter, W.M. and Glover, J.S. (1963) The preparation of ^{131}I-labelled human growth hormone of high specific radioactivity. Biochem. J. 89, 114–123.

Habeeb, A.F.S.A. (1966) Determination of free amino groups in proteins by trinitrobenzenesulfonic acid. Anal. Biochem. 14, 328–336.

Hartman, N.R., Johns, D.G. and Mitsuya, H. (1990) Pharmacokinetic analysis of dextran sulfate in rats as pertains to its clinical usefulness for therapy of HIV infection. AIDS Res. Hum. Retroviruses 6, 805–812.

Huppes, W., De Geus, B., Zurcher, C. and Van Bekkum, D. (1992) Acute human vs. mouse graft vs. host disease in normal and immunodeficient mice. Eur. J. Immunol. 22, 197–206.

Jansen, R.W., Molema, G., Harms, G., Kruijt, J.K., Van Berkel, T.J.C., Hardonk, M.J. and Meijer, D.K.F. (1991a) Formaldehyde treated albumin contains monomeric and polymeric forms that are differently cleared by endothelial and Kupffer cells of the liver: evidence for scavenger receptor heterogeneity. Biochem. Biophys. Res. Commun. 180, 23–32.

Jansen, R.W., Molema, G., Pauwels, R., Schols, D., De Clercq, E. and Meijer, D.K.F. (1991b) Potent in vitro anti-human immunodeficiency virus-1 activity of modified human serum albumins. Mol. Pharmacol. 39, 818–823.

Jansen, R.W., Olinga, P., Harms, G. and Meijer, D.K.F. (1993a) Pharmacokinetic analysis and cellular distribution of the anti-HIV compound succinylated human serum albumin (Suc-HSA) in vivo and in the isolated perfused rat liver. Pharm. Res. 10, 1611–1614.

Jansen, R.W., Schols, D., Pauwels, R., De Clercq, E. and Meijer, D.K.F. (1993b) Novel, negatively charged, human serum albumins display potent and selective in vitro anti-human immunodeficiency virus type 1 activity. Mol. Pharmacol. 44, 1003–1007.

Kageyama, S., Anderson, B.D., Hoesterey, B.L., Hayashi, H., Kiso, Y., Flora, K.P. and Mitsuya, H. (1994) Protein binding of human immunodeficiency virus protease inhibitor KNI-272 and alteration of its in vitro antiretroviral activity in the presence of high concentrations of proteins. Antimicrob. Agents Chemother. 38, 1107–1111.

Kuipers, M.E., Swart, P.J., Hendriks, M.M.W.B. and Meijer, D.K.F. (1995) Optimization of the reaction conditions for the synthesis of neoglycoprotein-AZT-monophosphate conjugates. J. Med. Chem. 38, 883–889.

Kuipers, M.E., Huisman, J.G., Swart, P.J., de Béthune, M.-P., Pauwels, R., Schuitemaker, H., De Clercq, E. and Meijer, D.K.F. (1996) Mechanism of anti-HIV activity of negatively charged albumins: biomolecular interaction with the HIV-1 envelope protein gp120. J. Acquir. Immun. Defic. Syndr. Hum. Retrovirol. 11, 419–429.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with the folin phenol reagent. J. Biol. Chem. 193, 265–275.

McCune, J.M., Namikawa, R., Shih, C.-C., Rabin, L. and Kaneshima, H. (1990) Suppression of HIV infection in AZT-treated SCID-hu mice. Science 247, 564–566.

Molema, G. and Meijer, D.K.F. (1992) Optimization of the synthesis of neoglycoprotein-AZTMP conjugates. Influence on stability, hepatic clearance and anti-HIV activity. In: Anonymous delivery of the anti-HIV drug azidothymidine (AZT) to T-lymphocytes with neoglycoprotein carriers, pp. 91–104. Thesis/Dissertation, University of Groningen, The Netherlands.

Mosier, D.E., Gulizia, R.J., Baird, S.M., Wilson, D.B., Spector, D.H. and Spector, S.A. (1991) Human immunodeficiency virus infection of human-PBL-SCID mice. Science 251, 791–794.

Pinter, A., Honnen, W.J. and Tilley, S.A. (1993) Conformational changes affecting the V3 and CD4-binding domains of human immunodeficiency virus type 1 gp120 associated with *env* processing and with binding of ligands to these sites. J. Virol. 67, 5692–5697.

Proost, J.H. (1985) Application of a numerical deconvolution technique in the assessment of bioavailability. J. Pharm. Sci. 74, 1135–1136.

Proost, J.H. (1987) Critical evaluation of the determination of bioavailability by numerical deconvolution. Ph.D. Thesis, University of Groningen, The Netherlands.

Proost, J.H. and Meijer, D.K.F. (1992) MW/Pharm, an integrated software package for drug dosage regimen calculation and therapeutic drug monitoring. Comput. Biol. Med. 22, 155–163.

Schutten, M., Tenner-Racz, K., Racz, P., Van Bekkum, D.W. and Osterhaus, A.D.M.E. (1996) Human antibodies that neutralize primary HIV-1 in vitro do not provide in vivo protection. J. Gen. Virol., 77(8), 1667–1675.

Swart, P.J. and Meijer, D.K.F. (1994) Negatively-charged albumins: a novel class of polyanionic proteins with a potent anti-HIV activity. Antiviral News 2, 69–71.

Swart, P.J., Schutten, M., Smit, C., Van Amerongen, G., Proost, J.H., Osterhaus, A.D.M.E. and Meijer, D.K.F. (1996a) Pharmacokinetics of succinylated serum albumin in wistar rats and cynomolgus monkeys: implications for dosage regimen in the therapy of HIV-infection. Drug Del. 3, in press.

Swart, P.J., Beljaars, E., Smit, C., Pasma, A., Schuitemaker, H. and Meijer, D.K.F. (1996b) Comparative pharmacokinetics, immunologic and hematologic studies on the anti-HIV-1 compounds succinylated and aconitylated HSA. J. Drug Targeting, 4, 109–116.

Swart, P.J., Sun, C.S., Kuipers, M.E., Asuncion, C., Josephs, S., Smit, C. and Meijer, D.K.F. (1996c) Antagonism of heparin on in vitro anti-HIV efficacy of succinylated human serum albumin. Submitted for publication.

Takakura, Y., Fujita, T., Furitsu, H., Nishikawa, M., Sezaki, N. and Hashida, M. (1994) Pharmacokinetics of succinylated proteins and dextran sulfate in mice: implications for hepatic targeting of protein drugs by direct succinylation via scavenger receptors. Int. J. Pharm. 105, 19–24.

Takami, M., Sone, T., Mizumoto, K., Kino, K. and Tsunoo, H. (1992) Maleylated human serum albumin inhibits HIV-1 infection in vitro. Biochim. Biophys. Acta 1180, 180–186.

Yahi, N., Fantini, J., Baghdiguian, S., Mabrouk, K., Tamalet, C., Rochat, H., Van Rietschoten, J. and Sabatier, J.-M. (1995) SPC3, a synthetic peptide derived from the V3 domain of human immunodeficiency virus type 1 (HIV-1) gp120, inhibits HIV-1 entry into $CD4^+$ and $CD4^-$ cells by two distinct mechanisms. Proc. Natl. Acad. Sci. USA 92, 4867–4871.

Yarchoan, R., Mitsuya, H. and Broder, S. (1990) Immunological issues in anti-retroviral therapy. Immunol. Today 11, 327–333.