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Preparation and characterization of conjugates of (modified) human serum albumin and liposomes: drug carriers with an intrinsic anti-HIV activity

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Abstract

Human serum albumin (HSA) derivatized with *cis*-aconitic anhydride (Aco-HSA) that was earlier shown to inhibit replication of human immunodeficiency virus type 1 (HIV-1), was covalently coupled to conventional liposomes, consisting of phosphatidylcholine, cholesterol and maleimido-4-(*p*-phenylbutyryl)phosphatidylethanolamine, using the heterobifunctional reagent *N*-succinimidyl-*S*-acetylthioacetate (SATA). The amount of HSA that could be coupled to the liposomes depended on derivatization of the HSA and ranged from 64.2 ± 9.2 μg HSA/μmol total lipid for native HSA to 29.5 ± 2.7 μg HSA/μmol total lipid for HSA in which 53 of the ϵ amino groups of lysine were derivatized with *cis*-aconitic anhydride (Aco_{53}-HSA). Incorporation of 3.8 mol% of total lipid of a poly(ethylene glycol) derivative of phosphatidylethanolamine (PEG-PE) in the liposomes resulted in a lower coupling efficiency of Aco-HSA. The elimination and distribution of the liposomal conjugates in rats in vivo was largely dependent on the modification of the HSA coupled to the liposomes. With native HSA-liposomes, more than 70% of the conjugate was still found in the blood plasma 30 min after i.v. injection in rats, while at this time Aco-HSA-liposomes were completely cleared from the circulation. The rapid clearance of conventional Aco-HSA-liposomes was due to a rapid uptake into the liver and could be considerably decreased by incorporating PEG-PE in the liposomal bilayer. After 3 h 60% of Aco-HSA-PEG-liposome conjugates were found in the blood. In an in vitro anti-HIV-1 assay, the 50% inhibitory concentrations (IC_{50}) for Aco_{39}-HSA-liposomes and Aco_{53}-HSA-liposomes expressed as protein weight, were 2.87 μg/ml and 0.154 μg/ml, respectively. When PEG-PE was incorporated, the Aco_{53}-HSA-liposomes retained anti HIV-1 activity (IC_{50}: 3.13 μg/ml). The possibility to modulate the residence time in the bloodstream of Aco-HSA-liposomes and the potent anti-HIV-1 activity of these conjugates, may allow the development of an intrinsically active drug carrier system. By incorporating anti HIV-1 drugs such as AZT into such liposomes a drug delivery system can be designed that might act simultaneously on the virus/cell binding by virtue of the coupled Aco-HSA and on the RNA/DNA transcription of the HIV-1 replication cycle through the nucleoside analogue.

Keywords: Liposome; Serum albumin; *cis*-Aconitic anhydride; Antiviral; HIV-1; SATA; (Human)

1. Introduction

Earlier we and others showed that human serum albumins (HSA) can be made highly negatively charged by derivatization of the ϵ amino groups of lysine (through reaction with the anhydrides of succinic acid or *cis*-aconitic acid). These polyanionic proteins display a selective in vitro anti-human immunodeficiency virus type 1 (HIV-1) activity [1–3]. Especially HSA that has reacted with *cis*-

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aconitic anhydride (Aco-HSA) proved to be a very potent inhibitor of HIV-1-induced cytopathogenicity [1]. The mechanism of action of these compounds is at the level of the binding and fusion of the virus with the target cells. The negatively charged HSA's have been proposed as potential carriers for 3'-azido-3'-deoxythymidine (AZT)-like drugs to T-lymphocytes and monocyte/macrophages. Such preparations may provide inhibition of HIV replication at the level of virus/cell fusion, syncytium formation as well as RNA reverse transcription [4]. AZT in the monophosphate (AZT-MP) form can be coupled directly to modified albumins yielding 1 to 6 molecules of AZT-MP per protein molecule [4]. Another approach to combine nucleoside analogues or other drugs with the negatively charged albumins, is by coupling these proteins to liposomes, which thus may function as high-capacity carriers for the chosen antiviral agent.

Liposomes have been proven to be a suitable delivery system for various kinds of therapeutics, including immune modulators, cytostatics, virostatics and antimicrobial agents [5–9]. Liposomes have also been used as carriers for anti-HIV-1 nucleoside analogues [10] and for prodrugs of nucleoside analogues [11,12]. These and other studies have shown that the antiviral activity of liposomal anti-HIV-1 nucleoside analogues can be preserved or even enhanced as compared to the free drug, while also reduced toxicity has been reported for such formulations [13]. Furthermore, the in vivo behaviour of liposomes can be modulated by incorporation of compounds like ganglioside GM1 or lipophilic derivatives of poly(ethylene glycol) to provide for a prolonged blood circulation time [14,15].

Several methods have been described for the covalent coupling of proteins to liposomes [16,17]. A widely used method, developed in our Institute, is based upon the reaction of sulfhydryl groups of thiolated proteins with maleimide residues incorporated in liposomes [18].

In this paper we describe the preparation of liposome conjugates of native HSA and of HSA, partially derivatized with *cis*-aconitic anhydride. Succinimidyl-*S*-thioacetate (SATA) was used to covalently couple the proteins to conventional or PEG-containing liposomes. The conjugates were chemically characterized and their disposition in rats was subsequently investigated. To verify whether the Aco-HSA liposome conjugates had antiviral activity and could be potentially used for a dual attack on the HIV-1 life cycle, we determined the effect of HSA-coupled liposomes on HIV-1 replication in vitro.

2. Materials and methods

2.1. Materials

Egg yolk phosphatidylcholine (PC), and maleimido-4-(*p*-phenylbutyryl)phosphatidylethanolamine (MPB-PE) were purchased from Avanti Polar lipids (Birmingham AL, USA). Human serum albumin fraction V (HSA) was obtained from the Central Laboratory of the Red Cross (Amsterdam, The Netherlands). Cholesterol (Chol), *N*-succinimidyl-*S*-acetylthioacetate (SATA) and *cis*-aconitic anhydride were from Sigma (St. Louis MO, USA). Iodine-125 (^{125}I) and [^{3}H]cholesteryloleyl ether were obtained from Amersham (Buckinghamshire, UK). Poly(ethylene glycol)-distearoylphosphatidylethanolamine (PEG-DSPE), synthesized from poly(ethylene glycol) with an average molecular weight of 1900 and distearoylphosphatidylethanolamine, was a generous gift of Liposome Technology (Menlo Park, CA, USA) through Dr. T.M. Allen. All other chemicals were analytical grade or the best grade available. Polycarbonate filters for liposome extrusion were obtained from Costar (Cambridge, MA, USA).

2.2. Methods

2.2.1. Preparation of cis-aconitic anhydride modified human serum albumin (Aco-HSA)

Ten mg of HSA were dissolved in 10 ml 0.2 M K_2HPO_4, pH 8.0. To obtain partially derivatized HSA's, variable amounts of solid *cis*-aconitic anhydride were added and the solution was stirred until all *cis*-aconitic anhydride was dissolved. The pH was kept at 8–8.5, using 3 M NaOH. Modified HSA was purified by Sephadex G-50 filtration. The modified albumins were characterized by protein determination according to Lowry [19] and estimation of free ϵ amino groups according to Habeeb [20]. The relative net charge of the modified albumins and the percentage monomers and dimers were determined as described before [1]. Shortly, this was done using a fast protein liquid chromatography (FPLC) system equipped with a mono-Q anion exchange column (Pharmacia; Uppsala, Sweden) or a Superose-12 column (Pharmacia), respectively. HSA was produced in which 39.0 ± 2.0 (mean $\pm$ S.D. of three batches) of the 60 ϵ amino groups were derivatized with *cis*-aconitic anhydride (Aco_{39}-HSA), and another in which 52.7 ± 1.5 (mean $\pm$ S.D. of three batches) of the ϵ amino groups were derivatized (Aco_{53}-HSA).

2.2.2. Liposome preparation

Lipids from stock solutions of PC, Chol and MPB-PE in chloroform/methanol (9:1), were mixed in a molar ratio of 23:16:1, dried under reduced nitrogen pressure, dissolved in cyclohexane and lyophilized. The lipids were hydrated in HN-buffer (10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), 135 mM NaCl, pH 6.7), under mechanical agitation. The liposomes were then sized by repeated extrusion (13 times) through polycarbonate filters (Costar, Cambridge MA, USA), pore size 50 nm, using a high pressure extruder (Lipex, Vancouver, Canada). When appropriate, 0.04 mol PEG_{1900}-DSPE (PEG) was added to the lipid mixture per mol of phospholipid. When required, liposomes were radiolabeled with a tracer amount of [^{3}H]cholesteryloleyl ether, which was included in the

lipid mixture during liposome preparation. Phospholipid phosphorus of each liposome preparation was assessed by phosphate assay after perchloric acid destruction [21]. Total liposomal lipid concentrations were adjusted for the amount of cholesterol present in the liposome preparations. Size and size distribution of the liposomes were determined by dynamic laser light scattering with a Nicomp model 370 submicron particle analyzer (NICOMP particle sizing systems, Santa Barbara, CA, USA). The diameter of the liposome preparations was obtained from the volume distribution curves produced by the particle analyzer.

2.2.3. Coupling of HSA or Aco-HSA to liposomes

HSA or Aco-HSA was coupled to MPB-PE liposomes by a sulfhydryl-maleimide coupling technique as described before [18], with minor modifications. Briefly, free sulfhydryl groups were introduced in HSA or Aco-HSA, as described by Duncan et al. [22], using *N*-succinimidyl-*S*-acetylthioacetate (SATA) as a heterobifunctional reagent. After separation of free SATA from the protein by gel permeation chromatography, acetylthioacetate-(Aco)-HSA was deacetylated by addition of 100 μl of a freshly prepared solution of 0.5 M hydroxylamine-HCl, 0.5 M Hepes, 25 mM EDTA, pH 7.0 per ml of protein solution and free sulfhydryl groups were determined according to Ellman [23]. After deacetylation, the thioacetyl-(Aco)-HSA was allowed to react for 4 h at room temperature with the MPB-PE containing liposomes in a ratio of 3 mg of protein per 10 μmol total lipid. *N*-Ethylmaleimide (8 mM in HN buffer) was added to cap unreacted sulfhydryl groups [18]. Liposomes were separated from unconjugated protein by flotation on a metrizamide gradient. 2 ml of the liposome mixture were mixed with 2.5 ml of a 60% metrizamide solution containing 10 mM Hepes, 6.7 mM KCl and 1.2 mM $CaCl_2$, pH 7.6. This solution was gently overlaid with 0.7 ml HN buffer and then centrifuged at $150000 \times g$, 4°C, in a Beckman SW 50.1 swing out rotor. The liposomes were collected between the buffer and the metrizamide layer, after which the separation procedure was repeated. The protein-liposome conjugates were then extensively dialyzed against HN buffer, pH 7.4. Control liposomes were prepared from the same lipid mixture but, instead of being incubated with (Aco)-HSA, they were incubated with cysteine in a molar amount twice that of MPB-PE to block reactive maleimido groups on the liposome surface. The (Aco)-HSA-liposome conjugates were characterized by determining protein [24] and phospholipid phosphorus content [21] and by particle size analysis. In some experiments HSA was labeled with ^{125}I as described by McFarlane [25] before starting the coupling procedure. Experiments with the (Aco)-HSA-liposome conjugates were performed within 1 week after preparation.

2.2.4. Serum disappearance, liver uptake and tissue distribution of liposomes

When in vivo experiments were performed with a duration of 30 min, male Wag-Rij rats (200–250 g) were anaesthetized by intraperitoneal injection of 20–25 mg sodium pentobarbital. Radiolabeled liposomes were injected via the penile vein and the abdomen was opened. As 100% value for the injected dose the actual amount of radioactivity injected was taken (corrected for radioactivity remaining in the needle, syringe and tube). At the indicated times, blood samples were taken from the inferior vena cava and allowed to clot for 60 min, at 4°C. The samples were centrifuged (5′ at $13000 \times g$). The total amount of radioactivity in the serum was calculated using the equation: serum volume (ml) = (0.0219 × body weight (g)) + 2.66 [26]. At the indicated times, liver lobules were excised and weighed. The total amount of liver tissue tied off did not exceed 15% of the total liver weight. Radioactivity in the liver was corrected for radioactivity present in serum at the time of sampling (85 μl serum/g of wet weight) [27]. When indicated, other tissues were removed and weighed at the end of the experiment. The tissues were homogenized using a Potter Elvehjem tube. Radioactivity was determined after solubilization of 0.4 ml of the homogenate in 100 μl 10% SDS and 4 ml scintillation cocktail. Radioactivity in the tissues was corrected for radioactivity originating from their plasma contents [27]. When experiments were performed with PEG-liposomes, rats were injected via the penile vein under light diethylether anesthesia. At the indicated time points blood samples were taken from the tail vein. At 3 h the rats were anesthetized and liver and spleen were removed and treated as described above.

2.2.5. Cells and virus

MT-4 cells, a T_4 lymphocyte cell line harbouring human T-cell lymphotropic virus type-1 (HTLV-1) [28], were used for the anti-HIV-1 assay. The MT-4 cells were grown in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum and 20 μg/ml gentamycin. The cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. Every 3–4 days, cells were centrifuged and seeded at $2 \cdot 10^5$ cells/ml in new culture flasks. At regular time intervals, the cells were analyzed for the presence of mycoplasma and were consistently found to be mycoplasma free. HIV-1 (strain HTLV-III_B) [29] was obtained from the culture supernatant of persistently HIV-1 infected HUT-78 cells. The virus titer of the supernatant was determined in MT-4 cells. The virus stock was stored at −70°C until used.

2.2.6. Antiviral assay

Antiviral activity of the test compounds was assessed by measurement of viability of MT-4 cells that had been infected or not infected with HIV-1 and exposed to various concentrations of the test compounds. The viability of the cells was examined spectrophotometrically by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method as described in more detail previously [30]. The cytotoxicity of the compounds was also monitored by the MTT assay.

Table 1
Characterization of conventional liposomes and PEG-liposomes

Protein coupled	mol SATA/ mol HSA	Liposome diameter (nm)	Protein/lipid ratio (μg/μmol TL)
Conventional liposomes			
none	–	74.7±1.1	–
HSA	5.4±0.7	131.1±6.5	64.2±9.2
Aco_{39}-HSA	4.5±0.6	85.8±1.5	35.2±3.7
Aco_{53}-HSA	4.8±0.6	92.0±4.3	29.5±2.7
PEG-liposomes			
none	–	77.4±1.4	–
Aco_{39}-HSA	4.5±0.6	96.3±8.5	20.3±1.9
Aco_{53}-HSA	4.8±0.6	87.8±2.6	22.9±2.3

The liposomes were characterized as described in Section 2. TL is total lipid. The data are presented as means±S.E. of 4–12 liposome preparations.

2.2.7. Statistical evaluation

Statistical significance of differences was evaluated by a two-tailed unpaired Student's *t*-test.

3. Results

3.1. Synthesis and characterization of (Aco)-HSA-liposomes

(Aco)-HSA was coupled to MPB-PE-containing liposomes with a diameter of 74.7 nm after extrusion through 50-nm polycarbonate filters (Table 1). When 3.8 mol% of total lipid PEG-DSPE was incorporated into the liposomes, a diameter of 77.4 nm was obtained after extrusion. Coupling of the different HSA preparations to the liposomes led to a significant increase of the particle diameter ($P < 0.05$) in all cases. Coupling of the heterobifunctional reagent SATA to the proteins resulted in the introduction of 4.5 to 5.4 sulfhydryl groups. Per albumin molecule the number of sulfhydryl groups introduced in the protein tended to decrease with the degree of derivatization with *cis*-aconitic anhydride. The amount of protein that could be coupled to the liposomes was therefore higher for native HSA than for Aco-HSA. For native HSA-liposomes a number 45 HSA molecules per liposome was calculated, assuming a molecular weight of HSA of 69 000 and assuming that 1 μmol liposomal lipid contains 48 000 liposomes [31]. Derivatization of HSA with either 39 molecules or 53 molecules of *cis*-aconitic anhydride did not significantly affect the amount of protein that could be coupled to either liposome type. However, slightly more Aco-HSA could be coupled covalently to conventional liposomes as compared to the PEG-liposomes.

The liposome conjugates were stable for at least 4 weeks at 4°C, with respect to size and to the amount of protein coupled. The in vivo stability of the conjugates was determined by comparing the liver uptake and serum decay of HSA-liposome conjugates which were either labeled in the lipid moiety with [^{3}H]cholesteryloleyl ether or in the protein moiety with ^{125}I (Fig. 1). The in vivo behaviour of the conjugates was independent of the type of label. Control liposomes, without coupled protein, showed a slower blood clearance and hepatic uptake.

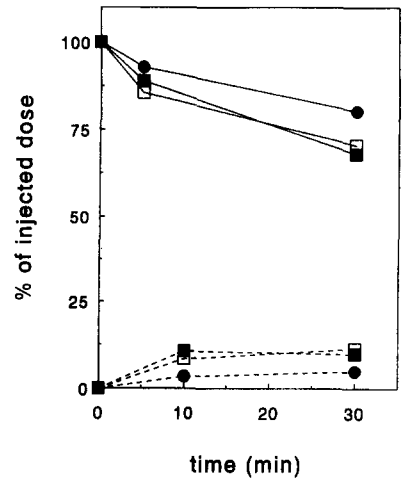

Fig. 1. Serum disappearance and liver uptake of control liposomes (●) and HSA-liposomes (■,□). Liposomes, which were labeled in the lipid moiety with [^{3}H]cholesteryloleyl ether (·,■) or in the protein moiety with ^{125}I (□), were injected into anesthetized rats. Radioactivity in serum (————) and liver (---) samples was determined as described in Section 2. Values are means of at least two rats.

3.2. Effect of coupled Aco_{39}-HSA and Aco_{53}-HSA on the serum disappearance of liposomes

The plasma disappearance of conventional control liposomes, Aco_{39}-HSA-liposomes and Aco_{53}-HSA-liposomes is shown in Fig. 2. Injection of either one Aco-HSA-liposome preparation resulted in a fast clearance of these

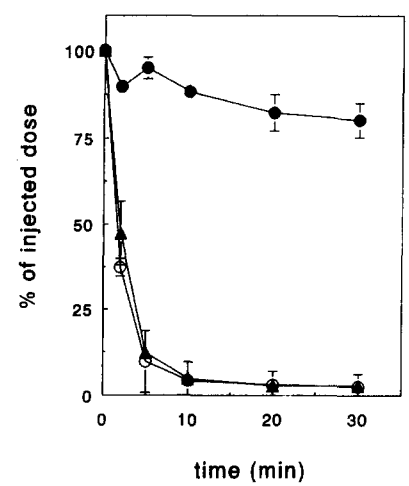

Fig. 2. Serum disappearance of control liposomes (●), Aco_{39}-HSA-liposomes (○) and Aco_{53}-HSA-liposomes (▲). [^{3}H]Cholesteryloleyl ether-labeled liposomes were injected into anesthetized rats. Blood samples were taken and radioactivity was determined as described in Section 2. Values are means±S.D. of 3–5 rats.

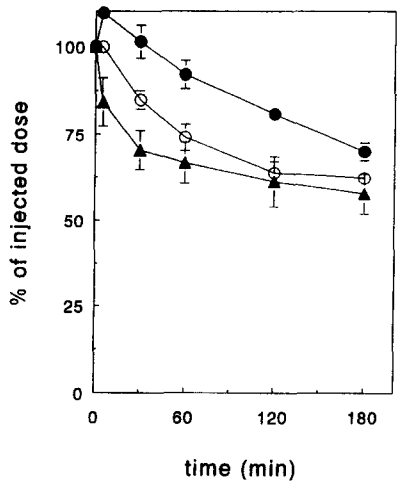

Fig. 3. Serum disappearance of control PEG-DSPE-liposomes (●), Aco_{39}-HSA-PEG-liposomes (○) and Aco_{53}-HSA-PEG-liposomes (▲). [^{3}H]Cholesteryloleyl ether-labeled liposomes were injected into anesthetized rats. Blood samples were taken and radioactivity was determined as described in Section 2. Values are means ± S.D. of 3–5 rats.

conjugates, as compared to control liposomes. At 10 min after injection, more than 95% of both the Aco_{39}-HSA and Aco_{53}-HSA-liposomes had disappeared from the blood plasma, while with control liposomes 88% was still present. There was no significant difference in clearance between Aco_{39}-HSA and Aco_{53}-HSA-liposomes. Incorporation of 3.8 mol% of total lipid PEG-DSPE in the liposomes could largely overcome the fast clearance from the circulation of conventional Aco-HSA-liposomes (Fig. 3). At 30 min after injection 85% of the injected dose of Aco_{39}-HSA-PEG-liposomes and 70% of Aco_{53}-HSA-PEG-liposomes were still present in the blood. In the first 30 min after injection of the conjugates, there was a significant difference in the plasma levels of Aco_{39}-HSA-PEG-liposomes and Aco_{53}-HSA-PEG-liposomes ($P < 0.005$), while after this time point the clearance of the two conjugates from the plasma was comparable up to 3 h. After 3 h of circulation about 60% of the Aco-HSA-PEG-liposomes was still found in the plasma, while about 70% of the control liposomes was recovered from the blood.

3.3. Tissue distribution of Aco-HSA-liposomes

The [^{3}H]cholesteryloleyl ether-labeled Aco-HSA-liposomes were mainly recovered in the liver and to a lesser extent in the spleen (Table 2). Other organs tested for radioactivity, such as kidney, lung and heart, did not show any significant uptake of Aco-HSA-liposomes (results not shown). At 30 min after i.v. injection of the conventional Aco_{39}-HSA- and Aco_{53}-HSA-liposomes, 80% was taken up by the liver, while the spleen accounted for 3% of the injected dose of these liposomes. When PEG-DSPE was incorporated in the Aco-HSA-liposomes, the liver remained the main site of uptake. However, at 3 h after administration of Aco_{39}-HSA- or Aco_{53}-HSA-PEG-liposomes, only 22% and 27% of the injected dose were found liver-associated, respectively. Spleen uptake of Aco-HSA-PEG-liposomes was lower than of control PEG-liposomes, but in the same range as that found for Aco-HSA-liposomes. Control PEG-liposomes were taken up by the spleen to a two-fold larger extent than by the liver, in line with earlier observations [32]. The total recovery of radioactivity from the tissues and fluids studied was always higher than 85% of the injected dose.

Table 2
Tissue distribution of conventional (Aco-HSA)-liposomes and (Aco-HSA-PEG)-liposomes

Liposome composition	Time (min)	Distribution (% of injected dose)		
		spleen	liver	serum
C-lip	30	4.6 ± 3.3	4.9 ± 5.6	80.2 ± 4.9
Aco_{39}-HSA-lip	30	2.9 ± 0.5	79.8 ± 18.3	2.5 ± 3.6
Aco_{53}-HSA-lip	30	3.1 ± 1.5	79.6 ± 20.0	2.8 ± 3.4
PEG-lip	180	9.1 ± 0.9	4.2 ± 2.1	70.0 ± 2.6
Aco_{39}-HSA-PEG-lip	180	3.2 ± 0.3	21.9 ± 0.9	62.3 ± 1.8
Aco_{53}-HSA-PEG-lip	180	2.6 ± 0.7	26.6 ± 3.3	57.7 ± 5.8

[^{3}H]Cholesteryloleyl ether-labeled liposomes were injected into anesthetized rats. Radioactivity in the different tissues was determined at the indicated time after injection as described in Section 2. Data are presented as means ± S.D. of 3–5 experiments. C-lip is control liposome.

3.4. Anti-HIV-1 activity of Aco-HSA-liposomes

Table 3 shows the antiviral activities of Aco_{39}-HSA, Aco_{53}-HSA and the different Aco-HSA liposome conjugates tested against HIV-1, in vitro. The IC_{50} values of the conventional Aco_{39}-HSA and Aco_{53}-HSA liposomes were 2.87 and 0.15 μg of protein/ml, respectively. Free Aco_{39}-HSA and Aco_{53}-HSA were 3 to 4 times more effective against HIV-1 replication in MT4 cells than the liposomal conjugates. Incorporation of PEG-DSPE in the liposomes resulted in a significant loss of antiviral activity of the Aco-HSA liposome conjugates, but with Aco_{53}-HSA-PEG-liposomes still an IC_{50} of 3.13 μg of protein/ml was obtained. Aco_{39}-HSA-PEG-liposomes did not show appre-

Table 3
Inhibitory effects of the compounds on HIV-1-induced cytopathogenicity

Compound	IC_{50} (μg protein/ml)	CC_{50} (μg protein/ml)	SI
HSA	> 250	> 250	> < 1
Aco_{39}-HSA	1.080	> 250	> 234
Aco_{53}-HSA	0.037	> 250	> 7011
Aco_{39}-HSA-lip	2.865	> 4	> 52–126
Aco_{53}-HSA-lip	0.154	> 12	> 53–277
Aco_{39}-HSA-PEG-lip	n.a.	–	–
Aco_{53}-HSA-PEG-lip	3.131	> 8	> 3

IC_{50} is the concentration needed to obtain 50% inhibition of virus-induced pathogenicity. CC_{50} is the concentration needed for 50% reduction of cell viability due to the compounds. SI is the selectivity index (CC_{50} / IC_{50}). n.a., not active (no anti-HIV-1 activity could be detected). Data are mean values of 2–4 determinations from two separate experiments.

ciable anti-HIV-1 activity. Control liposomes without Aco-HSA coupled, did not show any anti-HIV-1 activity (results not shown). The conjugates did not appear to be cytotoxic to MT4 cells at the concentrations used in the anti-HIV-1 assay. However, the cell toxicity (CC_{50}) and the selectivity index (SI) of the liposomal formulations could not be accurately determined, since the limited amount of protein that can be coupled to the liposomes did not allow testing of high protein concentrations in the cytotoxicity assay.

4. Discussion

In this report we describe the preparation and characterization of a liposomal drug carrier system with a potent intrinsic anti-HIV-1 activity. HSA and negatively charged Aco-HSA were covalently coupled to small type liposomes using the heterobifunctional reagent *N*-succinimidyl-*S*-acetylthioacetate. This is a well established method for coupling proteins to liposomes, based upon the reaction of thiolated proteins and liposomal maleimido-4-(*p*-phenylbutyryl)phosphatidylethanolamine [18,33,34]. The number of sulfhydryl groups that could be introduced in HSA or Aco-HSA was reproducible. Thiolation of Aco_{53}-HSA, in which 53 of the free ϵ-amino groups of lysine were derivatized with *cis*-aconitic anhydride, still allowed the introduction of 4.8 sulfhydryl molecules in this protein. This indicates that SATA is also very suitable for coupling highly derivatized proteins to liposomes. The amount of protein that could be coupled to the liposomes was significantly lower for the Aco-HSA than for native HSA. This may be due to the relatively strong negative charge of Aco-HSA [1], which may cause electrostatic repulsion. Incorporation of PEG-DSPE in the liposomes resulted in a slightly lower coupling efficiency of Aco-HSA. This is probably caused by steric hindrance by PEG at the liposome surface. Taking in account the serum decay and liver uptake patterns of HSA-liposomes, which were labeled either with ^{125}I in the protein moiety or with [^{3}H]cholesteryloleyl ether in the lipid moiety, it can be concluded that the covalent bond between the liposome and the coupled protein remains intact in the circulation.

Both Aco_{39}-HSA- and Aco_{53}-HSA-liposomes are rapidly cleared from the blood, while control liposomes show a very slow plasma disappearance. The rapid removal of the Aco-HSA-liposomes from the blood stream is associated with a high liver uptake. Spleen uptake of the liposomes was not affected by coupling of Aco-HSA. Low amounts of negatively charged succinylated HSA (Suc-HSA) [35] and Aco-HSA (Swart, P.J. et al., unpublished data) have also been shown to be taken up rapidly by the liver. This further substantiates that the in vivo behaviour of the Aco-HSA-liposomes is determined predominantly by the coupled protein and not by the lipid part of the conjugate.

Incorporation of phospholipid derivatives of PEG in liposomes has been shown to prolong the blood circulation time of liposomes [15]. Inhibition of the specific and/or non specific interaction between PEG modified liposomes and the mononuclear phagocyte system have been invoked to explain these favourable characteristics. At 3 h after injection of Aco_{39}-HSA-PEG- and Aco_{53}-HSA-PEG-liposomes blood concentrations were only 10% lower than the blood levels of control PEG-liposomes at that time. This indicates that incorporation of 3.8 mol% of total lipid PEG_{1900}-DSPE in the liposomes can largely overcome the effect of Aco-HSA on the residence time in the blood stream. Similar to what we observed with the conventional liposomes, the clearance of Aco-HSA-PEG-liposomes is mainly due to liver uptake of the conjugates. However, as compared to control PEG-liposomes the uptake by the spleen is significantly lower for Aco-HSA-PEG-liposomes than for control PEG-liposomes. This might be explained by the active involvement of the scavenger removal system in rat liver. Negatively charged proteins, including negatively charged HSA, have been demonstrated to be taken up very efficiently by the liver through scavenger receptors on liver endothelial cells and Kupffer cells [36]. When Aco-HSA-PEG-liposomes are accommodated by such scavenger receptors, this may cause a shift from splenic to hepatic uptake as compared to control PEG-liposomes.

Recently, it has been shown that Aco-HSA, with an IC_{50} of 0.02 μg/ml, is one of the most potent in vitro anti-HIV-1 agents described to date [1]. Furthermore Aco-HSA does not show unwanted side effects, such as anticoagulant activity, which has been described for dextran sulfate, a polyanion which also exhibits anti-HIV-1 activity [1]. The antiviral effect of Aco-HSA is rather specific for HIV-1 and the mechanism of action appears to be at the level of virus-cell binding and particularly on the fusion of the virus with the cell membrane. It has been postulated that these negatively charged albumins interact with the gp 41 envelope glycoprotein or may bind to the V3 loop of gp 120 [4]. These results were obtained with Aco-HSA in which all 60 amino groups of lysine were derivatized with *cis*-aconitic anhydride. The present study shows that the partially derivatized Aco_{39}-HSA and Aco_{53}-HSA still display a potent anti-HIV-1 activity of 1.08 μg/ml and 0.04 μg/ml, respectively. The observed difference in anti-HIV-1 activity between fully derivatized HSA and partially derivatized HSA indicates that the activity of these proteins is dependent on the degree of derivatization. This finding is in agreement with data which show that the anti-viral effect of negatively charged proteins correlates directly with the density of negative charges [3].

After coupling to conventional liposomes, the acylated proteins retain their anti-HIV-1 activity, although the antiviral activity decreases. When PEG-DSPE was incorporated in the liposomal bilayer no anti-HIV-1 activity could even be detected for Aco_{39}-HSA-PEG-liposomes, but for Aco_{53}-HSA-PEG liposomes still an IC_{50} of 3.13 μg/ml

was found. Since there is no significant physicochemical difference between Aco_{39}-HSA-PEG liposomes and Aco_{53}-HSA-PEG liposomes we suggest that the difference in antiviral activity is due to differences in acylation, while the decrease in anti-HIV activity as compared to conventional Aco-HSA liposomes may be caused by steric hindrance of the poly(ethylene glycol) on the surface of the liposomes.

With Aco_{53}-HSA-PEG-liposomes a dual attack on the life cycle of HIV-1 might be accomplished: firstly, on the binding/fusion of the virus with the cell membrane by means of the coupled Aco-HSA and, secondly, on the reverse transcriptase by including anti-HIV-1 nucleoside analogues. Apart from a potential dual attack on virus replication, there is evidence of synergistic effects of polyanionic compounds and nucleoside analogues [37,38]. It is of interest that in a previous report [39] liposomal preparations were described with an anti-HIV activity in which charge modification was not obtained by coupling of polyanionic proteins, but through inclusion of negatively charged lipids. The present results confirm the ability of net negatively charged liposomes to inhibit HIV-1 replication in vitro.

Since Aco_{53}-HSA-PEG liposomes have a long circulation time in blood, this conjugate may prove to be attractive as carriers for anti-HIV-agents to T-lymphocytes and monocytes/macrophages. Macrophages have been shown to endocytose polyanionic molecules including acylated proteins. Recent studies also indicate the presence of binding sites for negatively charged proteins on T-lymphocytes [4]. Recently, it was reported that both liver parenchymal and sinusoidal cells can be infected by HIV-1 in vivo [40]. In general, liposomes are avidly taken up by the liver, especially by the macrophages. Therefore, since both conventional as well as Aco-HSA-PEG-liposomes are, at least to some extent, taken up by the liver, they may be suitable carriers for the targeting of anti-HIV-1 drugs to various liver cells. We are currently investigating the mechanisms by which liver cell types take up Aco-HSA-liposome conjugates.

In conclusion, the procedure for coupling Aco-HSA to liposomes is reproducible and the conjugates seem to be stable after injection in vivo. The possibility to modulate the in vivo disposition of Aco-HSA-liposomes combined with the anti-HIV-1 activity of these conjugates, allows the development of a potent drug carrier system with a dual anti HIV-1 activity. Currently, studies are in progress in our laboratories to determine possible synergistic antiviral effects of anti-HIV-1 nucleoside analogues and Aco-HSA-liposomes as their delivery system.

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References

[1] Jansen, R.W., Schols, D., Pauwels, R., De Clercq, E. and Meijer, D.K.F. (1993) Mol. Pharmacol. 44, 1003–1007.
[2] Takami, M., Sone, T., Mizumoto, K., Kino, K. and Tsunoo, H. (1992) Biochim. Biophys. Acta 1180, 180–186.
[3] Jansen, R.W., Molema, G., Pauwels, R., Schols, D., De Clercq, E. and Meijer, D.K.F. (1991) Mol. Pharmacol. 39, 818–823.
[4] Molema, G. and Meijer, D.K.F. (1994) Adv. Drug Deliv. Rev. 14, 25–50.
[5] Daemen, T., Veninga, A., Roerdink, F.H. and Scherphof G.L. (1986) Cancer Res 46, 4330–4335.
[6] Fidler, I.J. and Kleinerman, E.S. (1994) Adv. Drug Deliv. Rev. 13, 325–340.
[7] Gabizon, A., Catane, R., Uziely, B., Kaufman, B., Safra, T., Cohen, R., Martin, F., Huang, A. and Barenholz, Y. (1994) Cancer Res. 54, 987–992.
[8] Ho, R.J.Y., Rouse, B.T. and Huang, L. (1987) J. Biol. Chem. 262, 13973–13978.
[9] Karlowsky, J.A. and Zhanel, G.G. (1992) Clin. Infect. Dis. 15, 654–667.
[10] Zelphati, O., Degols, G., Loughrey, H., Leserman, L., Pompon, A., Puech, F., Maggio, A.F., Imbach, J.L. and Gosselin, G. (1993) Antiviral Res. 21, 181–195.
[11] Van Borssum Waalkes, M., Fichtner, I., Dontje, B., Lemm, M., Becker, M., Arndt, D. and Scherphof, G.L. (1992) J. Microencaps. 9, 335–346.
[12] Hostetler, K.Y., Richman, D.D., Carson, D.A., Stuhmiller, L.M., Van Wijk, G.M.T. and Van den Bosch, H. (1992) Antimicrob. Agents Chemother. 36, 2025–2029.
[13] Phillips, N.C. and Tsoukas, C. (1992) Blood 79, 1137–1143.
[14] Woodle, M.C. and Lasic, D.D. (1992) Biochim. Biophys. Acta 1113, 171–199.
[15] Allen, T.M. (1994) Adv. Drug Deliv. Rev. 13, 285–309.
[16] Wright, S. and Huang, L. (1986) Adv. Drug Deliv. Rev. 3, 343–389.
[17] Sunamoto, J. and Iwamoto, K. (1986) Crit Rev. Ther. Drug Carrier Syst. 2, 117–136.
[18] Derksen, J.T.P. and Scherphof, G.L. (1985) Biochim. Biophys. Acta 814, 151–155.
[19] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[20] Habeeb, A.F.S.A. (1966) Anal. Biochem. 14, 328–336.
[21] Böttcher, C.J.F., Van Gent, C.M. and Pries, C. (1961) Anal. Chim. Acta 24, 203–204.
[22] Duncan, R.J.S., Weston, P.D. and Wrigglesworth, R. (1983) Anal. Biochem. 132, 68–73.
[23] Ellman, G.L. (1959) Arch. Biochem. Biophys. 82, 70–77.
[24] Peterson, G.L. (1977) Anal. Biochem. 83, 346–356.
[25] McFarlane, A.S. (1958) Nature 182, 53.
[26] Bijsterbosch, M.K., Ziere, G.J. and Van Berkel, Th.J.C. (1989) Mol. Pharmacol. 36, 484–489.
[27] Caster, W.O., Simon A.B. and Armstrong, W.D. (1955) Am. J. Physiol. 183, 317–321.
[28] Miyoshi, I., Taguchi, H., Kubonishi, I., Yoshimoto, S. and Ohtsuki, Y. (1982) Gann. Monogr. 28, 219–228.
[29] Popovic, M., Sarngadharan, M.G., Read, E. and Gallo, R.C. (1984) Science 224, 497–500.
[30] Pauwels, R., Balzarini, J., Baba, M., Snoeck, R., Schols, D., Herdewijn, P., Desmyter, J. and De Clercq, E. (1988) J. Virol. Methods 20, 309–321.

[31] Enoch, H.G. and Strittmatter, P. (1979) Proc. Natl. Acad. Sci. USA 76, 145–149.
[32] Scherphof, G.L., Morselt, H. and Allen, T.M. (1994) J. Lipos. Res. 4, 213–228.
[33] Hutchinson, F.J., Francis, S.E., Lyle, I.G. and Jones, M.N. (1989) Biochim. Biophys. Acta 978, 17–24.
[34] Francis, S.E., Lyle, I.G. and Jones, M.N. (1991) Biochim. Biophys. Acta 1062, 117–122.
[35] Jansen, R.W., Olinga, P., Harms, G. and D.K.F. Meijer (1993) Pharmaceut. Res. 10, 1611–1614.
[36] Jansen, R.W., Molema, G., Harms, G., Kruijt, J.K., Van Berkel. Th.J.C., Hardonk, M.J. and Meijer, D.K.F. (1991) Biochem. Biophys. Res. Commun. 18, 23–32.
[37] Anand, R., Nayyar, S., Galvin, T.A., Merril, C.R. and Bigelow, L.B. (1990) AIDS Res. Hum. Retroviruses 6, 679–689.
[38] Schols, D., De Clercq, Witvrouw, M. Nakashima, H., Snoeck, R., Pauwels, R., Van Schepdael, A. and Claes, P. (1991) Antiviral Chem. Chemther. 2, 45–53.
[39] Konopka, K., Davis, B.R., Larsen, C.E. and Düzgüneş, N. (1993) Antiviral Chem. Chemother. 4, 179–187.
[40] Housset, C., Lamas, E., Courgnaud, V., Boucher, O., Girard, P.M., Marche, C. and Brechot, C. (1993) J. Hepatol. 19, 252–258.