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Pharmacokinetic Properties and Interactions with Blood Components of N^4-Hexadecyl-1-β-D-arabinofuranosylcytosine (NHAC) Incorporated into Liposomes

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Abstract

N^4-Hexadecyl-1-β-D-arabinofuranosylcytosine (NHAC) is a new lipophilic derivative of 1-β-D-arabinofuranosylcytosine (ara-C) with strong antitumour activity. The interactions of NHAC incorporated into small unilamellar liposomes of different compositions with blood components were evaluated.

In comparison with ara-C, NHAC is highly protected against deamination to inactive arabinofuranosyluracil (ara-U) in human plasma, resulting in only 2% conversion into ara-U after 4 h incubation at 37°C, whereas from ara-C more than 80% was deaminated. In in-vitro incubations with human blood, it was found that NHAC was transferred from the liposomes at about 47% efficiency to plasma proteins, particularly to albumin and to the high and low density lipoproteins. The remaining part of NHAC was bound to erythrocytes (50%) and to leucocytes (3%). The addition of poly(ethylene) glycol-modified phospholipids to the liposomes (PEG liposomes), which were composed of soy phosphatidylcholine and cholesterol (plain liposomes), did not significantly prevent the fast transfer of NHAC from the liposomes to the blood components. Pharmacokinetic studies in mice revealed that NHAC had biphasic kinetics in blood with a $t_{2\alpha}^{\frac{1}{2}}$ of 16 min and a $t_{2\beta}^{\frac{1}{2}}$ of 3·8 h when the drug was formulated in plain liposomes and a $t_{2\alpha}^{\frac{1}{2}}$ of 15 min and a $t_{2\beta}^{\frac{1}{2}}$ of 9·67 h in PEG liposomes, respectively. NHAC was predominantly distributed in the liver with 29% of the injected dose found after 30 min. However, no accumulation occurred in the liver and NHAC was eliminated with biphasic kinetics resulting in a $t_{2\alpha}^{\frac{1}{2}}$ of 53 min and a $t_{2\beta}^{\frac{1}{2}}$ of 11·8 h. In spleen, kidney and bone marrow the levels of NHAC remained low.

In summary, NHAC is highly resistant against deamination and rapidly transferred from the liposomes to the blood components, independently of the liposome compositions tested.

1-β-D-Arabinofuranosylcytosine (ara-C, Fig. 1) is an effective chemotherapeutic agent for the treatment of acute myelogenous leukaemia (Keating et al 1982; Gahrton 1983; Plunkett & Gandhi 1993). However, its usefulness is impaired by its rapid deamination to the biologically inactive metabolite 1-β-D-arabinofuranosyluracil (ara-U) (Ho & Frei 1971). Thus, to be effective for the therapy of leukaemias, ara-C must be administered either continuously for five days (Frei et al 1969) as a conventional low dose schedule of 100–200 mg m^{-2} or as a high dose regimen with 1–3 g m^{-2} (Momparler 1974; Bolwell et al 1988).

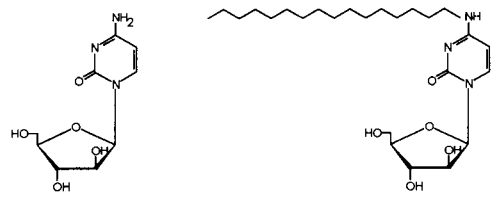

FIG. 1. Chemical structures of ara-C (left, molecular weight 243·2) and NHAC (right, molecular weight 467·7).

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To improve the cytotoxic activity of ara-C, combination therapies with deaminase inhibitors such as tetrahydrouridine (Marsh et al 1993; Kreis et al 1988) were clinically investigated.

In other approaches, the protection of ara-C from fast degradation and elimination was investigated by encapsulating the drug into liposomes (Mayhew et al 1976; Kobayashi et al 1977; Patel & Baldeschwieler 1984). However, protection against deamination and thus improvement of the antitumour activity of ara-C could not be increased significantly, mainly due to leakage of ara-C from the liposomes, which is caused by the well-characterized instability of liposomes in-vivo (Funato et al 1992). Recently, it was demonstrated that the modification of liposomes with poly(ethylene) glycol (PEG)-containing lipids greatly enhances their stability in the circulation (Allen et al 1991; Gabizon & Papahadjopoulos 1992). Allen et al (1992) compared the cytotoxic activity in mice of ara-C entrapped in PEG-modified small unilamellar liposomes (termed stealth liposomes) with liposomes of other lipid compositions as well as with the free drug. The resulting antitumour effect of ara-C encapsulated within long-circulating PEG liposomes was superior to the other liposome formulations and to the free drug. Kim et al (1993) demonstrated a prolongation of therapeutic ara-C concentrations in cerebrospinal fluid by encapsulation of

the drug into a preparation of multilamellar liposomes called DepoFoam.

The improvement of the cytostatic efficacy of ara-C can also be achieved by its chemical modification. A large number of N^4-derivatives of ara-C have been synthesized with the aim to increase the cytotoxic activity by protection against deamination and by altering the pharmacokinetic properties (Wempen et al 1968; Kanai & Ichino 1974; Rosowsky et al 1982). Whereas short-chain modifications of ara-C at the N^4-amino group resulted generally in weak enhancement of cytotoxicity (Aoshima et al 1976), lipophilic derivatives with long-chain fatty acids showed strong antitumour activity in murine tumour models (Kataoka & Sakurai 1980; Tsuruo et al 1980). In a previous study we reported that N^4-acyl-derivatives of ara-C incorporated into the membranes of small unilamellar liposomes were active against murine L1210 leukaemia and B16 melanoma at concentrations one-half to one-quarter that of unmodified ara-C administered as aqueous solution (Rubas et al 1986). However, the protection against enzymatic deamination to ara-U was only partially achieved with the acyl derivatives and suggested to be still insufficient in a pilot phase I/II study (Schwendener et al 1989). To obtain a compound with higher stability we synthesized the N^4-alkyl-ara-C derivative N^4-hexadecyl-1-β-D-arabinofuranosylcytosine (NHAC), (Schwendener & Schott 1992). Due to the very low solubility of NHAC in aqueous solutions this long chain alkyl derivative was incorporated into the lipid membranes of small unilamellar liposomes composed of soy phosphatidylcholine (SPC) and cholesterol to enable its parenteral application. These liposomal preparations exerted significantly higher cytotoxic activities in the L1210 leukaemia model at molar drug concentrations one-sixteenth those of ara-C. Furthermore, the derivative had a strong cytotoxic effect when administered as single dose therapy, suggesting a long-lasting drug effect.

It is known that single chain acyl compounds like fatty acids are not tightly anchored within the lipid bilayer of liposomes and that they are readily transferred to plasma proteins (Kamp & Hamilton 1993; Kleinfeld & Storch 1993; Richieri et al 1993). Assuming that NHAC, which has no amphiphilic properties and is not charged at physiological pH, may move through lipid membranes and be transferred to proteins at rates which are comparable with long-chain fatty acids, we investigated the interactions of NHAC with whole blood and its components in-vitro and in-vivo. Since we found that NHAC has a high cytotoxic activity in liposomes which lack the property of improved pharmacokinetics such as long blood circulation times and low uptake in the mononuclear phagocyte system (MPS), we compared the interactions of NHAC in-vitro and in-vivo with plasma proteins, erythrocytes and leucocytes in liposome preparations composed only of SPC and cholesterol (plain liposomes) with preparations containing poly(ethylene) glycol (2000)-dipalmitoylethanolamine (PEG(2000)-DPPE) in addition to SPC and cholesterol.

Furthermore we determined the pharmacokinetic properties in blood and the organ distribution of NHAC in mice, as well as the kinetics of [^{125}I]tyraminylinulin-labelled liposomes.

Materials and Methods

Chemicals

N^4-Hexadecyl-1-β-D-arabinofuranosylcytosine (NHAC) was synthesized as described before (Schwendener & Schott 1992). Soy phosphatidylcholine (SPC) was obtained from L. Meyer, Hamburg, Germany. Cholesterol (Fluka AG, Buchs, Switzerland) was recrystallized from methanol. (±)-α-Tocopherol, poly(ethylene)glycol-monomethyl ether (average molecular weight 2000, PEG(2000)) and all analytical grade buffer salts were from Merck, Darmstadt, Germany. 1,2-Dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE) was obtained from Sygena AG, Liestal, Switzerland. Tetrahydrouridine (THU) was obtained from the NCI, Bethesda, MD. Tritium-labelled ara-C (1·1 TBq mmol^{-1}, [5-^{3}H]ara-C) and custom-labelled N^4-hexadecyl-1-β-D-arabinofuranosylcytosine (0·189 GBq mmol^{-1}, [5-^{3}H]NHAC) were from Amersham Int., Amersham, UK.

Synthesis of PEG(2000)-DPPE

PEG(2000)-DPPE was synthesized according to Allen et al (1991) with minor modifications. The purification of the crude reaction product was achieved with a preparative Lichroprep RP-18 column (Lobar, Merck, Darmstadt, Germany) with 80% ethanol as eluent.

Preparation of liposomes

Small unilamellar liposomes were prepared by sequential filter extrusion of multilamellar liposome preparations through Nuclepore membranes (Sterico, Dietikon, Switzerland) of 0·4 and 0·1 μm using a Lipex extruder (Lipex Biomembranes Inc., Vancouver, Canada) (Hope et al 1985). The basic lipid composition for 1 mL liposomes (plain liposomes) was 20 mg soy phosphatidylcholine (SPC, 26 μmol), 3 mg cholesterol (7·7 μmol) and 0·13 mg (±)-α-tocopherol (0·3 μmol). Poly(ethylene)glycol-modified liposomes were obtained by addition of 6·9 mg PEG(2000)-DPPE (2·5 μmol) to the basic lipids (PEG(2000)-DPPE liposomes). The molar ratio of SPC : cholesterol : PEG(2000)-DPPE was 10 : 3 : 1. NHAC was added at 2·6 mg (5·56 μmol) and trace-labelled with [5-^{3}H]NHAC.

For the study of liposome kinetics in mice, ^{125}I-labelled tyraminylinulin (Sommerman et al 1984) was encapsulated in the aqueous inner volume of the liposomes by adding [^{125}I]tyraminylinulin to the lipid film before the preparation of the liposomes. Unencapsulated [^{125}I]tyraminylinulin was removed by ultrafiltration of the liposomes using Diaflo YM membranes (Amicon Corporation, Lexington, MA, 30 kDa cut-off) and subsequent gel filtration using a Sephadex G75 column (Pharmacia, Uppsala, Sweden, 200 × 10 mm).

All liposome preparations were sterilized by filtration through 0·2 μm filters (Gelman Sciences, Ann Arbor, MI). The mean diameter of the liposomes was determined by laser light scattering on a NICOMP 370 (Nicomp Instruments, Santa Barbara, CA) system with liposomes diluted to 0·2–0·7 μmol lipid mL^{-1} with phosphate buffer pH 7·4 (67 mM).

Deamination of ara-C and NHAC in human plasma

Ara-C (2 μM, trace labelled with [5-^{3}H]ara-C) dissolved in phosphate-buffered saline (PBS, 8 mM Na_2HPO_4, 1·5 mM

KH_2PO_4, 0·14 M NaCl, 2·6 mM KCl) or NHAC (2 μM, trace labelled with [5-^{3}H]NHAC) in plain liposomes were incubated with 300 μL human plasma at 37°C for different time periods up to 4 h. Tetrahydrouridine (2 μM) was added after incubation to inhibit further deamination and the probes ultrafiltrated using Amicon YM filters of 10 kDa cut-off. The filtrate was analysed for ara-U by ion-exchange HPLC on a Partisil SCX column and KH_2PO_4 (15 mM, pH 2·5) as elution phase (Spriggs et al 1987). The fractions containing ara-U were pooled and quantified by determination of [5-^{3}H]ara-U activity.

In-vitro distribution of NHAC in human blood

Fresh heparinized human blood from healthy donors was used for incubations with liposomal NHAC. The drug was incubated either in plain or PEG(2000)-DPPE liposomes in serial dilutions of 0·056, 0·56, 55·6, and 556 μmol NHAC with 10 mL blood. After incubation for 1 h at 37°C, the samples were transferred onto a Ficoll (d = 1·077 g mL^{-1}, Biochrom KG, Berlin, Germany) layer in a centrifuge tube and centrifuged for 30 min, 800 g. The concentration of NHAC in plasma was measured with three aliquots of 0·5 mL each of the supernatant plasma. The leucocytes were removed from the Ficoll-plasma interface and washed three times. The pelleted erythrocytes were washed accordingly and solubilized with 2–3 mL Soluene-350 (Packard)/2-propanol (1 : 1, v/v). These samples were incubated overnight at 50°C until a clear solution was obtained. Subsequently, the samples were bleached with 2–4 mL H_2O_2 35%. The concentration of NHAC was determined in all samples by measurement of the [5-^{3}H]NHAC activity by scintillation counting after the addition of 5 mL Hionic-Fluor (Packard Instruments, Groningen, The Netherlands). Leucocyte and erythrocyte numbers (cells mL^{-1}) were determined in a haemocytometer.

Binding capacity of NHAC in leucocytes and erythrocytes

The binding capacity of NHAC in leucocytes was measured in Ficoll-purified leucocytes obtained from a buffy coat (Swiss Red Cross SRC, Zürich). To 1 × 10^6 leucocytes 0·1, 1·0, 2·0, 10 and 100 nmol NHAC was added to give a final incubation volume of 0·5 mL. The samples were incubated for 1 h at 37°C. After three washings with 0·9% NaCl the leucocytes were lysed with 0·7 mL water and NHAC uptake determined by scintillation counting.

Additionally, the capacity of NHAC binding to erythrocytes obtained from an erythrocyte concentrate (SRC, Zürich) was measured in a concentration range of 0·001 to 1 nmol NHAC using as reference 1 × 10^6 erythrocytes suspended in 1 mL 0·9% NaCl. After 1-h incubation at 37°C and three washings with 0·9% NaCl, the erythrocytes were made permeable by addition of 2 mL water and 0·3 mL 2% Triton X-100 and centrifuged at 3000 g for 10 min, separated from haemoglobin and washed three times with 0·9% NaCl. For determination of NHAC in the supernatant the samples were bleached as described above.

In-vitro distribution of NHAC in plasma proteins

The distribution of liposomal NHAC in the different plasma proteins (albumin, high density lipoprotein (HDL), low density lipoprotein (LDL), very low density lipoprotein (VLDL)) was determined by gradient-ultracentrifugation (Chung et al 1980; Chapman et al 1981). Blood from a healthy donor was collected in an EDTA-tube (Vacutainer, Becton Dickinson) and centrifuged at 1500 g for 10 min. NHAC (556 nmol in 0·1 mL) in plain or PEG(2000)-DPPE liposomes was incubated with 0·9 mL plasma for 1 h at 37°C. A density gradient was formed in 5-mL ultracentrifuge tubes by adding 0·325 g KBr to 1 mL plasma (d = 1·21 g mL^{-1}) overlaid with 1 mL each of KBr/0·9% NaCl solutions of d = 1·063 g mL^{-1} and d = 1·019 g mL^{-1}, respectively. The tubes were then filled with 2 mL 0·9% NaCl (d = 1·006 g mL^{-1}). The probes containing NHAC in the two liposome formulations incubated in plasma and the reference liposomes without plasma as well as a plasma control were centrifuged for 24 h at 280 000 g at 20°C (Beckman SW 50·1 rotor). At the end of a run, the KBr gradient was separated into fractions of 0·05 mL by means of an injection needle which was carefully immersed to the bottom of the tubes. Ninety to 100 fractions were collected. The elution of the proteins was monitored at a continuous flow with a UV flow-detector (LKB Uvicord II) at 280 nm.

In-vivo interaction of NHAC with mouse blood

The in-vivo distribution of NHAC in plain and PEG(2000)-DPPE liposomes was studied by intravenous injection of 22·2 μmol liposomal NHAC kg^{-1} into three BDF1 (C57B1/6J × DBA/2J)F1 mice of 20 g average body weight. Five minutes after injection the mice were killed and the blood removed by heart puncture. The blood samples were centrifuged at 1500 g for 5 min. Total NHAC in the plasma fractions as well as NHAC bound to erythrocytes was determined by scintillation counting as described. To separate liposome-associated NHAC from plasma-bound drug, plasma aliquots (25 μL, corresponding to 1·5 mg protein) were chromatographed on an HPLC Biosep SEC 3000 column (300×4·6 mm) (Phenomenex, Torrance, CA) protected by a Biosep SEC guard column (30 × 4·6 mm). As mobile phase, phosphate buffer (67 mM, pH 7·4; supplemented with 0·01% sodium azide) was used at a flow rate of 0·15 mL min^{-1}. Detection was at 280 nm and 1-min fractions were collected. The concentration of NHAC per fraction was measured by scintillation counting.

Pharmacokinetics and organ distribution of NHAC and ^{125}I-labelled liposomes in mice

NHAC (5·0 mg NHAC per kg body weight) trace labelled with [5-^{3}H]NHAC (12 kBq per animal) in plain liposomes or in PEG(2000)-DPPE liposomes was injected intravenously into the tail vein of ICR mice (20–25 g, females, 12–15 weeks of age). After time periods ranging from 5 min to 24 h, groups of three mice were killed and blood, liver, spleen, kidney and bone marrow from femur and tibia were collected. Whole weighed organs were digested with 2–4 mL Soluene 350 tissue solubilizer at 40–50°C until total solubilization of the organs. Blood and bone marrow were solubilized with 2 mL of a 1 : 1 (v/v) mixture of Soluene 350 and isopropanol. The solubilized samples were bleached by dropwise addition of 0·5–1 mL hydrogen peroxide 30% and counted after addition of 10–15 mL Hionic-Fluor scintillation fluid. The total blood volume was calculated at 7·7% of the body weight and the concentrations of NHAC in liver,

spleen and kidney were corrected for blood contamination according to Allen (1989). Results are expressed as % of injected dose of NHAC in whole organs. The blood clearance half-lives were calculated using proFit software (Quantumsoft, Zürich, Switzerland).

The kinetics of the liposomes was determined by the injection of plain and PEG(2000)-DPPE liposomes of the usual compositions containing NHAC ($5\,mg\,kg^{-1}$) and [^{125}I]tyraminylinulin (12 kBq per animal) additionally encapsulated into the inner liposome volume. After time periods ranging from 5 min to 24 h, groups of three mice were killed, the blood collected and the concentration of [^{125}I]tyraminylinulin measured in a γ-counter (Packard).

Results and Discussion

The structural formulas of ara-C and NHAC are shown in Fig. 1. Deamination from ara-C and NHAC in human plasma is shown in Fig. 2. After 4 h incubation at 37°C ara-U formation from NHAC was less than $39\,pmol\,mL^{-1}$ plasma corresponding to 2% of the initial concentration, whereas a 42-fold amount of ara-U corresponding to 84% was formed from ara-C. Thus, the N^4-alkylation of ara-C with long alkyl chains provides an excellent protection against the transformation of the drug into the inactive ara-U.

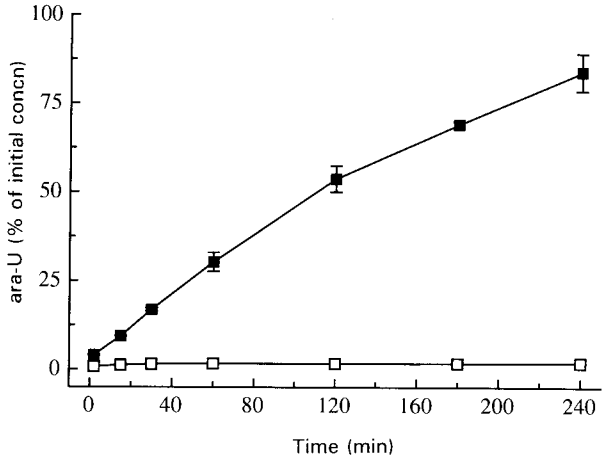

FIG. 2. Formation of ara-U in human plasma (300 μL) after incubation with 2 μM ara-C (■) or 2 μM NHAC (□) plain liposomes. The plasma samples were analysed for ara-U by HPLC using a Partisil SCX column and KH_2PO_4 (15 mM, pH 2·5) as elution phase. The fractions containing ara-U were pooled and counted for ^{3}H-activity.

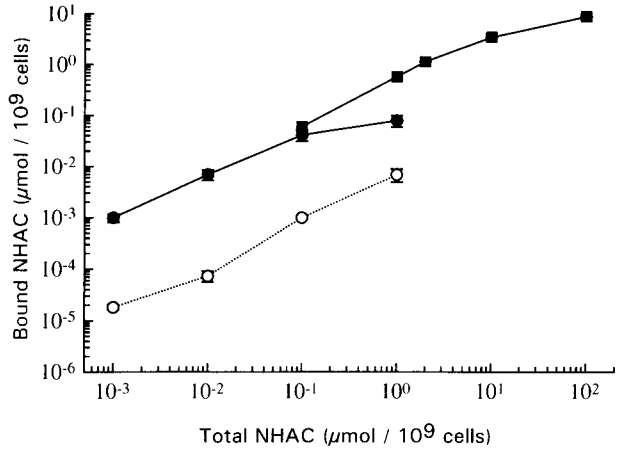

FIG. 3. Binding of NHAC in plain liposomes to human leucocytes (■), erythrocytes (●) and erythrocyte lysate (○). To samples of $1{\cdot}4 \times 10^6$ washed leucocytes, NHAC was added in the concentration range 0·14–140 nmol in 0·5 mL. The samples were incubated for 60 min at 37°C. The washed leucocytes were then lysed with 0·7 mL water and the cell-bound NHAC concentrations determined. Accordingly, $2{\cdot}8 \times 10^9$ erythrocytes were incubated with NHAC (2·8 nmol–2·8 mmol) in 1 mL for 60 min at 37°C. After centrifugation and lysis with 2% Triton X-100 the membranes and lysate were counted for [5-^{3}H]NHAC activity.

Concentration-dependent binding of NHAC in plain liposomes to isolated human leucocytes and erythrocytes is shown in Fig. 3. For comparison between leucocytes and erythrocytes the binding of NHAC was normalized to 10^9 cells, which does not reflect the true ratio in human blood, where the average numerical ratio between erythrocytes and leucocytes is 2500:1. Taking this ratio into account, the amount of total bound NHAC to erythrocyte membranes would, therefore, be 2500 times higher. The concentrations of NHAC found in the supernatant of permeable erythrocytes was about one hundredfold lower which may be explained by the lipophilic nature of the drug, demonstrating that NHAC is predominantly membrane bound and has no haemolytic properties. The binding of NHAC to erythrocytes and leucocytes was further evaluated in a Lineweaver–Burk plot. For erythrocytes, a K_m value of 0·33 μmol NHAC/10^9 cells and a V_{max} value of 0·21 μmol NHAC/10^9 cells h^{-1} was determined. NHAC binding to leucocytes was weaker with a K_m value of 16 μmol/10^9 cells and a V_{max} value of 10·35 μmol/10^9 cells h^{-1}.

To assess the distribution of NHAC in the main components of whole blood, we separated human blood after incubation of liposomal NHAC into plasma, leucocytes

Table 1. Distribution of NHAC in plasma, leucocytes and erythrocytes after in-vitro incubation with human blood. To 10 mL human blood NHAC in either plain or PEG(2000)-DPPE liposomes was added at different concentrations and incubated for 1 h at 37°C. Leucocytes were separated over Ficoll and washed three times with phosphate buffer. NHAC concentration was determined in plasma, leucocytes and erythrocytes. The values obtained from the NHAC distribution were normalized to 100%.

NHAC (μM)	NHAC in plain liposomes (% of initial concentration)			NHAC in PEG liposomes (% of initial concentration)		
	Plasma	Leucocytes	Erythrocytes	Plasma	Leucocytes	Erythrocytes
5·56	47 ± 2	2·7 ± 0·05	50 ± 3	46 ± 1	2·4 ± 0·1	52 ± 1
55·6	46 ± 4	1·8 ± 0·03	52 ± 2	43 ± 1	1·8 ± 0·05	55 ± 3
555·6	46 ± 2	1·5 ± 0·02	53 ± 4	48 ± 3	1·1 ± 0·01	51 ± 2

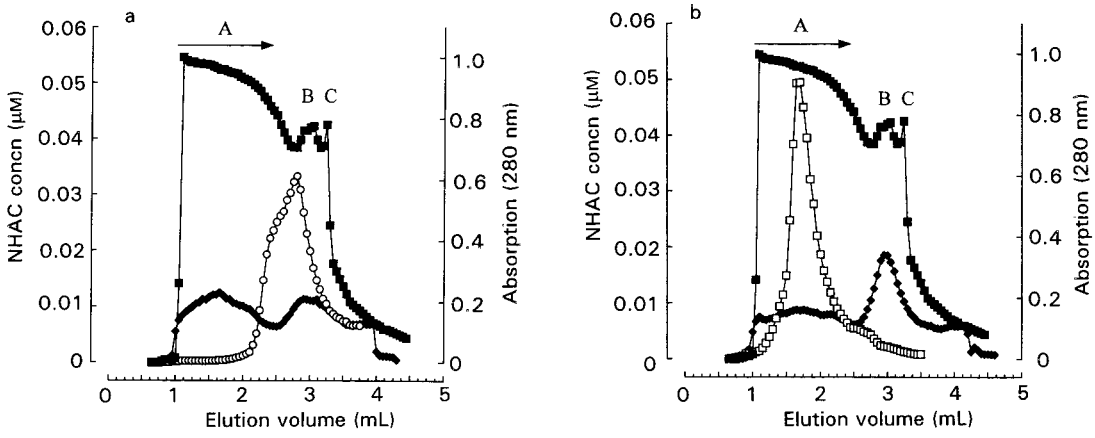

FIG. 4. Binding of NHAC in plain liposomes (a) and in PEG(2000)-DPPE liposomes (b) to plasma proteins and lipoproteins. NHAC-liposomes (0·1 mL, 5·5 mM) were incubated with fresh human plasma (0·9 mL) for 60 min at 37°C. The samples were overlaid on a KBr density gradient and centrifuged for 24 h at 280 000 *g*. Fractions (0·05 mL) of the centrifuged samples were collected from the bottom of the tubes and [^{3}H]NHAC activity and protein absorption at 280 nm determined. ■ Protein absorption, ○ plain reference liposomes, ◆ NHAC (μmol/0·05 mL eluate), □ PEG 2000-DPPE reference liposomes. A. Elution of plasma proteins and HDL, B. LDL peak, C. VLDL peak.

and erythrocytes. As summmarized in Table 1, NHAC is mainly bound to plasma proteins and to the erythrocyte membranes. Independently of the liposome composition, NHAC interacted with the blood components in a comparable fashion. The poly(ethylene)glycol hydration layer covering the surface of the PEG-modified liposomes does not seem to substantially alter the transfer of NHAC to the blood components. To further characterize the binding of NHAC to the plasma proteins, we incubated liposomal NHAC with human plasma and separated the proteins on a KBr gradient. Fig. 4 shows the protein-binding pattern of NHAC in plain liposomes (panel a) and in PEG(2000)-DPPE liposomes (panel b) to the fractions containing HDL plus albumin (A), LDL (B), and VLDL (C). For comparison, the distribution patterns of the liposomes and the proteins over the KBr gradient, monitored by measurement of the absorption at 280 nm, were superimposed on the binding patterns of NHAC. Whereas plain liposomes had a rather broad distribution over the fractions of intermediate density of the KBr gradient, the PEG-modified liposomes were concentrated as a narrow peak in fractions of higher densities (d = 1·07–1·21 g mL^{-1}).

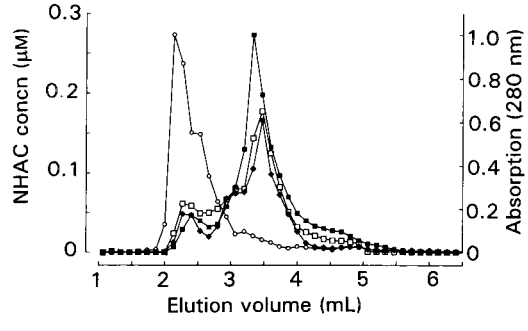

FIG. 5. Separation of liposome-associated and plasma-protein bound NHAC after intravenous administration to BDF$_1$ mice of plain and PEG(2000)-DPPE liposomes. Plasma was collected 5 min after injection of NHAC (22·2 μmol kg^{-1}) in plain liposomes. Separation was made on a Biosep SEC 3000 column with phosphate buffer (67 mM, pH 7·4) as eluent. The flow rate was 0·4 mL min^{-1} and fractions of 140 μL were collected and analysed for [5-^{3}H]NHAC activity and absorption at 280 nm, respectively. The figure shows a representative elution pattern of three individual experiments. ○ Plain liposomes as reference, ■ plasma proteins as reference, ◆ [5-^{3}H]NHAC from plain liposomes, □ [5-^{3}H]NHAC from PEG(2000)-DPPE liposomes.

The monitoring of the distribution of NHAC measured by [5-^{3}H]NHAC activity revealed for the incubation of plain NHAC-liposomes (Fig. 4a) a distribution over the plasma proteins with an average of 54% of the drug bound to HDL and albumin (A, fractions 0·9–2·75 mL), 39% to LDL (B, fractions 2·8–3·2 mL) and 7% to VLDL (C, fractions 3·25–4·25 mL). In contrast, binding of NHAC incubated in PEG(2000)-DPPE liposomes (Fig. 4b) with plasma was more prominent in the LDL (45%, B) and VLDL (12%, C) fractions. Correspondingly, binding to HDL and albumin (43%, A) was reduced.

In Fig. 5 the elution pattern of a plasma sample taken 5 min after intravenous injection into BDF$_1$ mice of 22·2 μmol kg^{-1} NHAC and the separation of liposome-associated and protein-bound NHAC from plain and PEG(2000)-DPPE modified liposomes by gel permeation HPLC is shown. NHAC remained associated in the PEG(2000)-DPPE liposomes at proportions 4–10% higher than in plain liposomes (Fig. 5). The differences of the in-vivo distribution of NHAC between the two liposome formulations are summarized in Table 2. Protein binding of NHAC ranged between 86 and 89% for the PEG(2000)-DPPE liposomes and between 86 and 96% for the plain liposomes, showing that in spite of the addition of PEG(2000)-DPPE to the liposomes, the major proportion of NHAC was transferred shortly after administration from the lipid membranes to the plasma proteins and the erythrocytes. At the concentrations of 0·22 and 2·22 μmol NHAC kg^{-1}, a slightly different distribution pattern between plain and PEG(2000)-DPPE liposomes was observed. Erythrocyte binding did not significantly differ between the two formulations, with the highest proportions of NHAC bound at the lowest incubation concentration. Binding to leucocytes could not be determined due to the small blood samples.

The blood levels of [^{125}I]tyraminylinulin-labelled liposomes and of NHAC in ICR mice are shown in Fig. 6. The addition

Table 2. In-vivo distribution of NHAC in plasma and erythrocytes 5 min after intravenous injection into mice. After separation of plasma and erythrocytes, plasma aliquots were chromatographed as shown in Fig. 5 to separate liposome-associated NHAC from plasma bound drug.

Injected dose NHAC (μmol kg^{-1})	Plasma (% NHAC)			Erythrocytes (% NHAC)	Recovery (% NHAC)
	Total bound	Liposome associated	Protein bound		
NHAC in plain liposomes					
0·22	29 ± 2	3·6 ± 1·4	96·4 ± 1·4	71 ± 2	9·6
2·22	45 ± 4	6·5 ± 0·9	93·5 ± 0·9	55 ± 7	11·4
22·2	57 ± 4	13·5 ± 1·8	86·5 ± 1·8	43 ± 1	15·5
NHAC in PEG liposomes					
0·22	37 ± 3	13·8 ± 3·1	86·1 ± 3·1	63 ± 8	12·6
2·22	42 ± 5	10·8 ± 1·0	89·2 ± 1·0	58 ± 7	11·0
22·2	51 ± 4	14·1 ± 2·7	85·9 ± 2·7	49 ± 5	9·5

of PEG(2000)-DPPE to the basic liposome composition resulted in a significant increase of the circulation half-life of the liposomes. With a $t_{\frac{1}{2}}$ of 9·2 h we obtained a half-life which is comparable with that reported by Allen et al (1992) for PEG-liposomes of similar composition. In contrast to the long lasting high blood levels of the PEG(2000)-DPPE liposomes, the plain liposomes were cleared from circulation at much faster rates, with a $t_{\frac{1}{2}\alpha}$ of 46 min and a $t_{\frac{1}{2}\beta}$ of 6·4 h. The determination of the blood levels of NHAC from both liposome formulations showed that the drug is rapidly released from the liposomes. With both liposome formulations, significant amounts of NHAC were distributed into other body compartments within the first hours after application (Table 3). While the blood concentration of NHAC given in plain liposomes was reduced after 24 h to less than 0·1% of the injected dose, the NHAC levels remained higher (1·17 ± 0·15% after 24 h) with the PEG(2000)-DPPE liposomes. The half-lives of NHAC in blood were $t_{\frac{1}{2}\alpha} = 16$ min and $t_{\frac{1}{2}\beta} = 3{\cdot}8$ h for plain liposomes and $t_{\frac{1}{2}\alpha} = 15$ min and $t_{\frac{1}{2}\beta} = 9{\cdot}7$ h for PEG(2000)-DPPE liposomes.

As summarized in Table 3, NHAC in PEG(2000)-DPPE liposomes was predominantly distributed into the liver, reaching the highest concentration with 29 ± 3·3% after 30 min. NHAC was not accumulated in liver tissue and the drug was eliminated from the liver with half-lives of $t_{\frac{1}{2}\alpha}$ of 53 min and a $t_{\frac{1}{2}\beta}$ of 11·8 h. The levels of NHAC in spleen and kidney remained below 5%. In the bone marrow, NHAC was found at levels of 0·1–0·06% (2–24 h) of the injected dose.

Table 3 shows the organ distribution of [^{125}I]tyraminylinulin-labelled PEG(2000)-DPPE liposomes. The liver concentrations increased from 2·6% after 30 min to 17% of the injected dose after 24 h. The levels of [^{125}I]tyraminylinulin detected in the spleen remained below 5%. In the kidneys, only 2·2–2·5% (2–24 h) of the injected dose of [^{125}I]tyraminylinulin was found, confirming that the liposomes retained their vesicular properties during circulation.

We have demonstrated that NHAC is highly resistant against deamination in human plasma and may, therefore, be administered at lower doses to achieve the same antitumour effects in-vivo (Schwendener & Schott 1992). The results of the pharmacokinetic studies indicated that NHAC is rapidly released from both liposome formulations after intravenous injection and that the modification of the liposomes with PEG(2000)-DPPE could not prevent the fast transfer of large amounts of NHAC from the liposome membranes to the blood components and to other organs during the first hour after administration. In a later phase, however, the PEG(2000)-DPPE liposomes were able to delay the further distribution and elimination of NHAC, resulting in 10-fold concentrations in the blood of ICR mice 24 h after intravenous administration. In summary, the

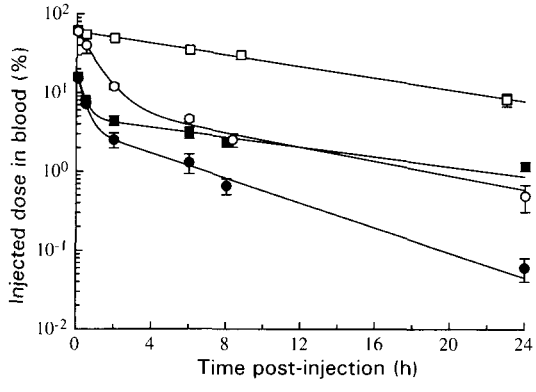

FIG. 6. Pharmacokinetics of plain liposomes, PEG(2000)-DPPE liposomes and NHAC in ICR mice after intravenous application. The liposome kinetics in blood were determined by [^{125}I]tyraminylinulin for plain (○) and PEG(2000)-DPPE) (□) liposomes. Correspondingly, the kinetics of NHAC from plain (●) and from PEG(2000)-DPPE (■) liposomes were determined by measurement of [5-^{3}H]NHAC.

Table 3. Organ distribution of NHAC administered in PEG(2000)-DPPE-modified liposomes and of [^{125}I]tyraminylinulin-labelled liposomes.

	NHAC (% injected dose)		
	0·5 h	8·0 h	24·0 h
Blood	8·1 ± 0·3	2·4 ± 0·3	1·2 ± 0·2
Liver	29·0 ± 3·3	7·9 ± 0·9	2·9 ± 0·02
Spleen	0·8 ± 0·04	0·4 ± 0·08	0·25 ± 0·03
Kidney	5·33 ± 0·3	3·1 ± 0·27	0·86 ± 0·18
	Tyraminylinulin (% injected dose)		
	0·5 h	8·0 h	24·0 h
Blood	55·0 ± 4·0	30·2 ± 1·2	8·3 ± 1·5
Liver	2·7 ± 0·04	12·0 ± 1·6	17·2 ± 2·0
Spleen	0·44 ± 0·07	0·9 ± 0·2	2·6 ± 0·9
Kidney	0·9 ± 0·18	2·6 ± 0·7	2·5 ± 0·6

overall effect of the liposome composition on the pharmacodynamic and pharmacokinetic properties of incorporated NHAC was only minor. Thus, the liposomes serve mainly as a pharmaceutical formulation to enable the solubilization and parenteral administration of NHAC in aqueous media.

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