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New lipophilic alkyl/acyl dinucleoside phosphates as derivatives of 3′-azido-3′-deoxythymidine: Inhibition of HIV-1 replication in vitro and antiviral activity against Rauscher leukemia virus infected mice with delayed treatment regimens

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Abstract

The antiretroviral activity of two new lipophilic derivatives of azidothymidine (AZT), N^4-hexadecyl-2′-deoxyribocytidylyl-(3′-5′)-3′-azido-2′,3′-deoxythymidine(N^4-hexadecyldC-AZT) and N^4-palmitoyl-2′-deoxyribocytidylyl-(3′-5′)-3′-azido-2′,3′-deoxythymidine(N^4-palmitoyldC-AZT) was evaluated in comparison to AZT. In vitro the drugs were tested in human immunodeficiency virus 1 (HIV-1) infected $CD4^+$ HeLa and H9 cells. The in vivo antiviral effect of these derivatives was analysed in Rauscher leukemia virus (RLV) infected mice. The derivatives were incorporated into small liposomes. In vitro both derivatives inhibited virus proliferation in both HIV-1 infected cell lines in a similar dose-responsive manner as AZT. In a plaque reduction assay, using HeLa cells, the IC_{50} values were 0.035 μM for AZT, 0.5 μM for N^4-hexadecyldC-AZT and 4.5 μM for N^4-palmitoyldC-AZT, whereas p24 antigen analysis on H9 cells gave IC_{50} values of 0.005 μM, 0.05 μM and 0.05 μM, respectively. RLV infected mice were treated with intermittent schedules i.p. or i.v. on days 1, 6, 11, and days 16 or 0, 3, 7, and 11 after infection. Regimens with further delayed drug application were on days 3, 7, and 11 and 7 and 11 only. While i.p. treatment with total doses of 380–1140 mg/kg free AZT resulted in 10–30% inhibition of RLV induced splenomegaly, the derivatives gave inhibitions

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of 37–94%. Late onset of treatment with the derivatives was significantly more effective as compared to free AZT. Intravenous treatment with N^4-hexadecyldC-AZT was effective, but with AZT was inactive. The discrepancy in antiviral activity of the AZT derivatives found between the in vitro and in vivo test systems emphasizes the importance of investigating the activity of drug derivatives in vivo.

Key words: HIV-1; Azidothymidine; Derivative; Liposome; Antiviral activity

1. Introduction

Azidothymidine (AZT) was the first drug licensed for the treatment of HIV-1 infections in humans. With the aim of increasing its antiviral activity, several investigators have converted this nucleoside analogue into a variety of lipophilic derivatives. AZT conjugated to monophosphate diacylglycerols showed in vitro anti-HIV-1 activities comparable to that of the parent drug (Steim et al., 1990). AZT derivatives containing a polar carbohydrate and an hydrophobic hexadecyl chain retained their anti-HIV-1 activity and displayed high affinity to lipid membranes (Henin et al., 1991). Ether lipids were linked to AZT and 2′,3′-dideoxyinosine (ddI) through phosphate and phosphonate linkages in order to alter the biological properties of the parent drugs, again with the aim to increase the antiviral efficacy of the nucleosides (Meyer et al., 1991; Piantadosi et al., 1991). Hostetler and coworkers prepared phospholipid analogues of AZT and other antiviral nucleosides which had anti-HIV-1 activity in vitro as liposome preparations (Hostetler et al., 1990; Hostetler et al., 1991; Hostetler et al., 1992).

Liposomes are ideally suited for the incorporation of such lipid-like antiviral compounds. Since they have a natural affinity to cells of the mononuclear phagocyte system (MPS) and to lymphoid tissues, they thus provide new possibilities for the delivery of antiretroviral drugs or prodrugs to the cells which represent virus reservoirs (Fox et al., 1991; Embretson et al., 1993; Pantaleo et al., 1993).

Drugs with unfavourable pharmacokinetic properties, such as short plasma half-lives or fast metabolic inactivation, are increasingly being prepared in liposome formulations. These liposomes are generally prepared following two distinct concepts. In the first, water-soluble compounds such as AZT (Phillips et al., 1991; Phillips and Tsoukas, 1992; Phillips, 1992), dideoxycytidine (Kim et al., 1990), phosphorylated nucleosides (Szebeni et al., 1990), antisense oligodeoxynucleotides (Renneisen et al., 1990) or antitumor and other antiviral agents (Gabizon, 1992; Gangemi et al., 1987; Hong and Mayhew, 1989) have been encapsulated into the inner aqueous volume of the lipid vesicles. Recently, it was shown that phosphorylated dideoxyuridine derivatives which were encapsulated into immunoliposomes could inhibit HIV-1 replication in vitro (Zelphati et al., 1993). The advantage of the encapsulation approach is that chemically unaltered molecules are used in these preparations. However, low encapsulation efficiencies and possible leakage of the entrapped molecules during storage may hamper the clinical application of such preparations. In the second approach, these disadvantages can be circumvented using

chemically modified active molecules. For instance, lipophilic derivatives of antiviral and antitumor drugs can be quantitatively integrated in a stable fashion into the lipid membranes of liposomes. We have demonstrated that lipophilic derivatives of cytosine arabinoside (ara-C), i.e., of N^4-oleyl-ara-C and N^4-hexadecyl-ara-C, exhibit cytostatic activities in murine tumor models (Rubas et al., 1986; Schwendener and Schott, 1992). A phase I/II clinical trial has been conducted with the former ara-C derivative (Schwendener et al., 1989). To alter the biological properties of AZT, we have chosen the latter approach. A new class of lipophilic dinucleotide derivatives were synthesized and examined for their antiviral properties in vitro and in vivo.

Infection with HIV causes variable, usually, slow degradation of immune functions. Shortly after an individual is infected by HIV, antibodies are produced which bind to circulating viruses. These antigen-antibody complexes are quickly cleared by cells of the MPS and, for those immune complexes appearing in the lymphatic circulation, deposition on the surface of follicular dendritic cells occurs. It has been suggested that, at least in the early stages of infection, the germinal centers of lymphoid tissues and helper T lymphocytes as well as macrophages serve as substantial reservoirs of HIV (Fox et al., 1991; Embretson et al., 1993; Pantaleo et al., 1993). Using the PCR technique, it was recently found that high levels of HIV-1 are in fact present in plasma during all stages of infection (Piatak et al., 1993). In view of the pathobiology of all stages of HIV infection, new therapeutic strategies must be developed that reach beyond the treatment regimens used today (Connolly and Hammer, 1992).

In this report, we analysed in vitro and in vivo the antiviral properties of liposomes containing two lipophilic derivatives of AZT, namely N^4-hexadecyl-2′-deoxyribocytidylyl-(3′-5′)-3′-azido-2′,3′-deoxythymidine (N^4-hexadecyldC-AZT) and N^4-palmitoyl-2′-deoxyribocytidylyl-(3′-5′)-3′-azido-2′,3′-deoxythymidine (N^4-palmitoyldC-AZT).

2. Materials and Methods

2.1. Drug synthesis

The new class of lipophilic dinucleoside phosphates containing AZT were obtained through the 3′7–5′ condensation of AZT with N^4-hexadecyl- or N^4-palmitoyl-2′-deoxyribocytidine. The lipophilic deoxyribocytidine derivatives were obtained as described (Schwendener and Schott, 1992). The detailed synthesis and chemical analysis of the AZT derivatives will be published elsewhere. The chemical structures of the AZT derivatives are shown in Fig. 1.

2.2. Liposome preparation

Small unilamellar liposomes (SUV) were prepared by filter extrusion (Hope et al., 1985). The basic lipid composition was soy phosphatidylcholine (SPC, L. Meyer, Hamburg, Germany), cholesterol and DL-α-tocopherol (Merck AG, Darmstadt, Germany) at molar ratios of 10:2:0.1. The lipid concentration was 20–60 mg SPC/ml and the concentration of the AZT derivatives varied between 5 and 12 mg/ml,

corresponding to 8–20 mol parts referred to SPC. These lipid-derivative mixtures were dissolved in methanol/methylene chloride (1:1, v/v, Merck) and after exhaustive evaporation of the organic solvents at 40°C small unilamellar liposomes were obtained by repeated sequential extrusion of lipid suspensions in phosphate buffer (PB, 67 mM, pH 7.4) through Nuclepor™ (Costar, Cambridge, MA) membranes of decreasing pore size (1.0–0.1 μm). Drug-free control liposomes with 60 mg SPC, 6 mg cholesterol and 0.3 mg DL-α-tocopherol per milliliter were prepared identically. All liposome preparations were sterile filtrated (0.45 μm Acrodisc, Gelman, Ann Arbor, MI) and stored at 4–7° C. Average liposome diameters and the vesicle population homogeneity were determined with a Nicomp 370 particle sizer (Particle Sizing Systems, Sta. Barbara, CA).

2.3. Micellar drug preparations

Due to their amphiphilic nature, both AZT derivatives are soluble in aqueous media by the formation of micelles. For the in vitro experiments, the derivatives were dissolved in PB at 5 mM and sterile filtrated through 0.2 μm Acrodisc filters.

2.4. Biological activity against HIV-1 infected cells

(a) Plaque reduction in CD4$^+$ HeLa 1022 cells. The plaque reduction immunoassay, developed by Chesebro and coworkers was used to test the activity of free AZT and the lipophilic AZT derivatives (Chesebro and Wehrly, 1988; Larder et al., 1990; Chesebro et al., 1991). Briefly, 5×10^5 CD4$^+$ Hela 1022 cells were seeded into 24-well plates in RPMI 1640 medium (GIBCO, Grand Island, NY) and incubated overnight at 37°C, 5% CO_2. In most experiments, the adhered cells were incubated with various concentrations of the antiviral substances for 18 h prior to infection. Before infection, the cells were treated for 20 min with 8 mg/ml DEAE dextran (Pharmacia, Uppsala, Sweden) in serum-free medium. The cells were infected for 2 h with a culture supernatant from HIV-1 (IIIB)-infected H9 cells containing 100–200 plaque-forming units per well. After infection, the cells were washed with medium and then incubated for 3 days in medium containing the antiviral drugs in micellar and liposomal formulations. Infected cells (foci) were identified immunologically using pooled sera from HIV-1-infected patients. 50% inhibitory concentrations (IC_{50}) were derived from plots of the percentage of foci obtained with the drug relative to that obtained without addition of the drug (Fig. 2A,B).

(b) Antiviral activity in HIV-1 infected H9 cells. N^4-hexadecyldC-AZT and N^4-palmitoyldC-AZT were analysed for their effect on the p24 antigen production in the supernatant of HIV-1 infected cells. H9 cells (Medical Research Council, AIDS Reagent Project) were infected with HIV-1 (IIIB) at 0.004 50% tissue culture infective doses per cell for 1–2 h at 37°C, extensively washed to remove virus, and then distributed in 48-well plates at 1.2×10^5 cells per well before addition of medium containing drugs or empty liposomes (0.08–8 μM SPC) as control. After 3 days incubation at 37°C and 5% CO_2, the antiviral activity was measured as the reduction of p24 antigen in the cell-free supernatant. The p24 antigen was measured in duplicate by an enzyme-linked immunoassay according to the manufacturer's instructions (NEN Research Products, Boston, MA). The data are expressed as per-

centages of antigen obtained without the addition of drug (Fig. 3). Uninfected drug-treated toxicity controls were maintained and routinely analysed for cell proliferation and viability by trypan blue exclusion method.

2.5. In vivo activity in the Rauscher leukemia virus (RLV) model

The RLV model, described by Ruprecht et al. for testing the antiviral activity of AZT, was used to evaluate the activity of the liposomal AZT derivatives (Ruprecht et al., 1986). Female Balb/c mice (Institut für Labortierkunde, University of Zürich) of 18–22 g average weight (3 or 7 mice/group) were infected intravenously with the virus corresponding to an average of $4–5 \times 10^4$ plaque forming units of RLV and then treated with various concentrations of lipophilic AZT derivatives in liposomes or with AZT dissolved in phosphate buffer (PB). For the assessment of toxic drug effects, groups of 3 uninfected mice per drug concentration were treated identically as the infected animals (Table 3). The drugs were administered either intraperitoneally (i.p.) or intravenously (i.v.) with intermittent schedules on days 1, 6, 11, 16 (Fig. 4 and Table 1) or on days 0 (4 h after infection), 3, 7, 11 after RLV infection (Table 2). Delayed treatment was started either on day 3 or day 7 after infection with an additional treatment on day 11 or on day 7 and 11 only (Table 2). The inhibition of splenomegaly was assessed on day 20 or 14 (Table 2), respectively, after infection by determination of spleen weights. The mice were anaesthetized with ether and bled by heart puncture. Per cent inhibition of splenomegaly was calculated using the formula $\{1\text{-}(x-y)/(c-n)\}100$ with x, mean spleen weight of virus infected and treated mice; y, that of uninfected and treated mice; c, that of virus infected control mice; and n, that of normal untreated mice (Ruprecht et al., 1986).

RLV-specific antigens (gp70env, p30gag) in spleen extracts and in serum were detected by immunoblotting using a polyclonal goat anti-RLV antiserum (NIH No. 75S000297) provided by A. Hügin, NIH, Bethesda, MD (Ruprecht et al., 1990a).

In a separate experiment, uninfected and RLV infected female Balb/c mice (5/group) were treated with drug-free control liposomes. For the i.p. day 1, 6, 11, and 16 treatment schedule the lipid concentration of 60 mg/dose, corresponding to the highest dose of 1140 mg/kg AZT given as N^4-hexadecyldC-AZT (Fig. 4) with a total lipid dose of 12 g/kg was used. The evaluation of effects on the spleens was made on day 20 as described.

2.6. Haematological toxicity

Blood samples from uninfected healthy Balb/c mice treated with N^4-hexadecyldC-AZT and AZT on days 1, 6, 11, and 16 (Fig. 4) were collected on day 20, the time of killing of the mice. The haematological parameters (Table 3) such as white blood cell count per μl (WBC), red blood cell count per μl (RBC) and haemoglobin (g/%) were determined with a Contraves Autolyzer 820 (Contraves AG, Zürich).

2.7. Statistical analysis

The effects of the AZT derivatives on inhibition of splenomegaly and on haema-

tological toxicity were compared statistically with those of AZT using the Student's *t*-test.

3. Results

3.1. Liposome size and stability

The mean diameters of the liposomes, containing the AZT derivatives at 5–12 mg/ml, ranged between 100 and 250 nm after repeated extrusion through membranes of 100 nm pore size. Empty control liposomes had a size of 94 ± 35 nm. The liposomes remained stable for more than 6 months, when stored at 4–7°C.

3.2. Susceptibilities of HIV-1 to AZT and lipophilic derivatives of AZT

The plaque-reduction assay in Hela $CD4^+$ clone 1022 cells (Chesebro et al., 1991) provided a good system to test the the new antiviral agents. The data presented in Figs. 2A and B show that both of the new derivatives exhibited a dose-response inhibition of HIV-1 replication. Whereas free AZT reduced the plaque formation by 50% (IC_{50}) at 0.035 μM, N^4-hexadecyldC-AZT and N^4-palmitoyldC-AZT caused a 50% inhibition at 0.5 μM and 4.5 μM, respectively. Both lipophilic derivatives were equally active in liposomal formulations or as micelles. The results presented in Figs. 2A and B were derived from experiments in which the cells were preincubated with the antiviral substances for 18 h prior to infection. Addition of the drug only after infection resulted in approx. 10-fold higher IC_{50}s for the lipophilic derivatives, however, the IC_{50} of free AZT remained the same (data not shown).

The antiviral activity of liposomal preparations containing N^4-hexadecyldC-AZT and N^4-palmitoyldC-AZT was additionally measured in HIV-1-infected H9 cells

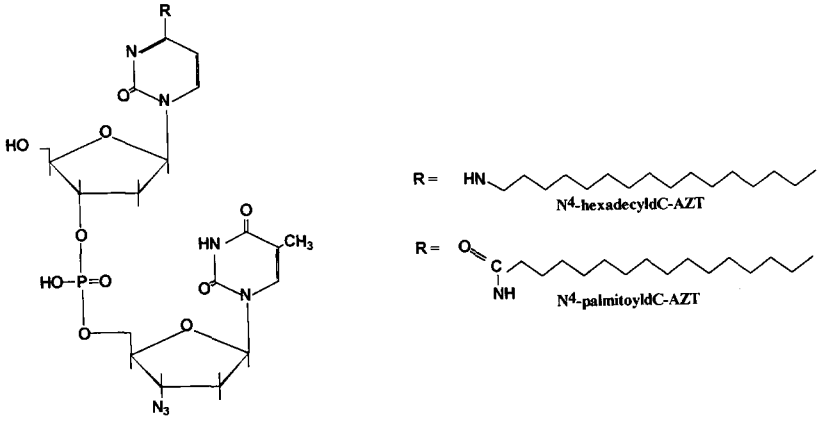

Fig. 1. Chemical structures of N^4-hexadecyl-2′-deoxyribocytidylyl-(3′-5′)-3′-azido-2′,3′-deoxythymidine (N^4-hexadecyldC-AZT, mol.wt. 779.9) and N^4-palmitoyl-2′-deoxyribocytidylyl-(3′-5′)-3′-azido-2′,3′-deoxythymidine (N^4-palmitoyldC-AZT, mol.wt. 793.9).

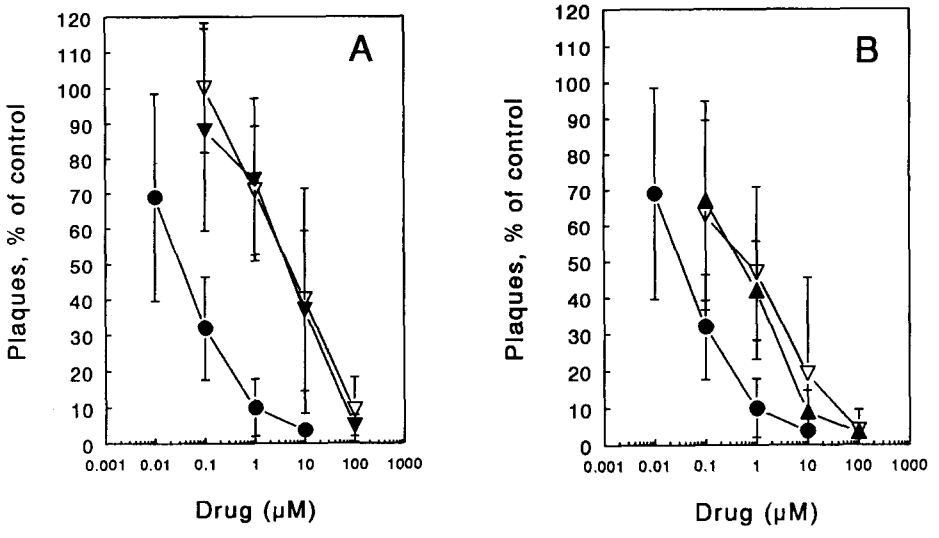

Fig. 2. Effect of free AZT, N^4-hexadecyldC-AZT and N^4-palmitoyldC-AZT in liposomal or micellar formulations on HIV-1 replication in HeLa 1022 cells. (A) N^4-hexadecyldC-AZT in micelles (open triangles) and in liposomes (closed triangles). (B) N^4-palmitoyldC-AZT in micelles (open triangles) and in liposomes (closed triangles). Free AZT is shown in both figures (closed circles). Values are means ± S.D. ($n = 3$).

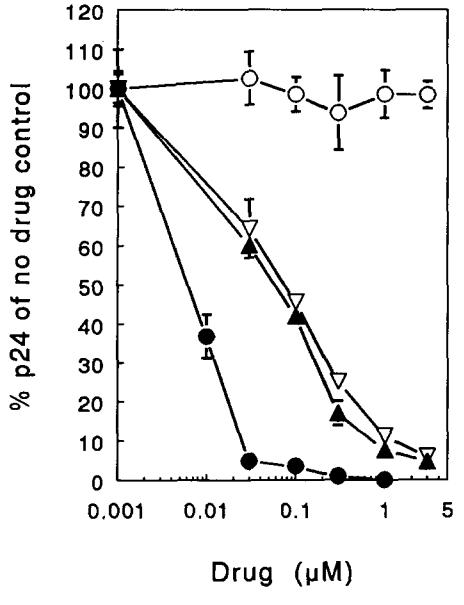

Fig. 3. Effect of free AZT, N^4-hexadecyldC-AZT and N^4-palmitoyldC-AZT in liposomes and empty liposomes on HIV-1 replication in H9 cells, measured by p24 antigen reduction. Free AZT (closed circles); N^4-hexadecyldC-AZT (open triangles); N^4-palmitoyldC-AZT (closed triangles) and empty liposomes (0.08–8 μM lipid concentration, open circles). Values are means ± S.D. ($n = 3$).

(Fig. 3). Similar to the plaque-reduction assay, the IC_{50} in H9 cells with free AZT was approx. 10-fold lower than that observed with the liposomal formulations. However, the actual drug concentrations required to achieve the 50% inhibition in this assay were lower for all drugs than those concentrations required in the plaque-reduction experiments (0.005 μM free AZT; 0.05 μM N^4-hexadecyldC-AZT and N^4-palmitoyldC-AZT). Empty liposomes, with lipid compositions corresponding to those with the lipophilic-AZT containing liposomes, were not cell toxic and were ineffective against HIV-1 infection at all concentrations (0.08–8 μM SPC) tested (Fig. 3).

3.3. In vivo activity in the Rauscher leukemia virus model

The results of the antiviral activity of liposomal preparations containing N^4-hexadecyldC-AZT and N^4-palmitoyldC-AZT are shown in Fig. 4 and Tables 1 and 2. Fig. 4 summarises the results of free AZT (filled bars), given i.p. in the concentration range of 380–1140 mg AZT/kg as total dose on days 1, 6, 11, and 16 after infection. AZT exerted a weak antiviral effect, whereas treatment with liposomal N^4-hexadecyldC-AZT (open bars) resulted in a more pronounced concentration-dependent antiviral effect. At the highest total dose of 3330 mg/kg N^4-hexadecyldC-AZT an inhibition of splenomegaly of 93.7% was achieved, which is significantly different ($P=0.00002$) from the 13.7% found with AZT. The total dose of 3330 mg N^4-hexadecyldC-AZT corresponds to 1140 mg AZT/kg. This is the same dose of AZT

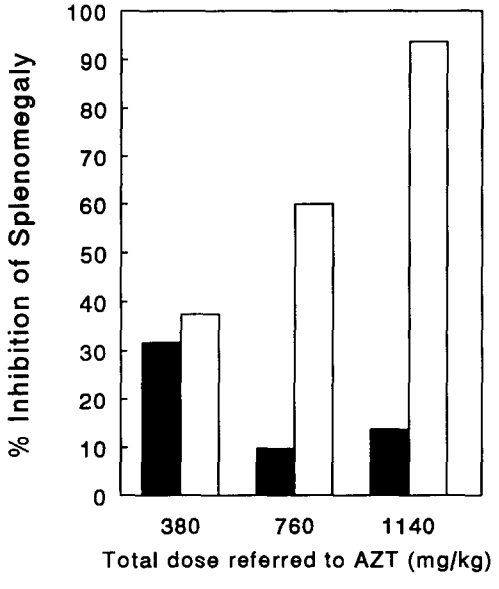

Fig. 4. Concentration-dependent inhibition of splenomegaly of RLV infected Balb/c mice after i.p. treatment on days 1, 6, 11, and 16 with N^4-hexadecyldC-AZT in liposomes and with free AZT. Free AZT (closed bars); N^4-hexadecyldC-AZT in liposomes (open bars).

Table 1
Intravenous treatment of RLV infected mice on days 1, 6, 11, and 16 with AZT and liposomal N^4-hexadecyldC-AZT

Drug preparations	Total dose		Spleen weight grams ± SD[a]	% Inhibition of splenomegaly
	mg/kg	mmol/kg		
AZT in phosphate buffer	380	1.42	1.99 ± 0.35	−5.9
N^4-hexadecyldC-AZT liposomes	1110	1.42	1.19 ± 0.19	48.1
Controls infected	–	–	1.86 ± 0.27	0
Controls uninfected	–	–	0.14 ± 0.05	–

[a]S.D., standard deviation, n = 7–10; evaluation on day 20.

as used by Ruprecht and coworkers who gave 20 mg AZT/kg i.p. every 8 h during 19 days resulting in an inhibition of splenomegaly of 94% (Ruprecht et al., 1986). Thus, the lipophilic N^4-hexadecyldC-AZT derivative appears to exert a similar antiretroviral effect with the intermittent treatment schedule as compared to either free AZT given daily every 8 h i.p. or given dissolved in the drinking water of the mice. A reduction in the total dose to 380 mg AZT/kg, corresponding to 1110 mg N^4-hexadecyldC-AZT per kg, resulted in 37.5% inhibition of splenomegaly. This was not significantly different ($P=0.79$) from 31.6% determined with the equal total concentration of free AZT, whereas at the dose of 760 mg/kg a significant difference ($P=0.01$) between the two drugs was found (see Fig. 4). The high concentrations of free AZT were less active, probably due to additional toxic effects. However, none of the treatments were lethally toxic.

The antiretroviral effect after i.v. treatment is documented in Table 1. A total dose of 1110 mg/kg N^4-hexadecyldC-AZT resulted in a significant ($P=0.001$) inhibition of RLV-induced splenomegaly, whereas AZT dissolved in buffer was ineffective. Higher intravenous doses were not applied because of the limited injection volumes.

In Table 2, we compared the antiretroviral effect of the two lipophilic AZT derivatives and AZT using 3 different intermittent i.p. treatment schedules with the evaluation of the inhibition of splenomegaly on day 14. One was initiated immediately on day 0 (4 hours after infection), and then continued on days 3, 7, and 11. The other two schedules were initiated several days after infection, i.e. on days 3, 7, and 11 or on days 7 and 11, respectively. It was assumed that the delayed schedule would mimic a more realistic case in which an infected individual is treated with an antiviral drug a long time after the initial infection. The two lipophilic AZT derivatives differ only in the chemical linkage of the lipophilic side chain to the cytidine moiety of the molecule (see Fig. 1). In the case of N^4-hexadecyldC-AZT, the linkage of the hexadecyl side chain to the cytidine moiety can be expected to be relatively resistant to enzymatic cleavage, whereas the acyl linkage of palmitic acid to the amino group of cytidine in N^4-palmitoyldC-AZT may be more susceptible. With the treatments on days 0, 3, 7, 11 and 3, 7 and 11, respectively, a large reduction in splenomegaly occurred with both AZT derivatives.

Table 2
Effect of delayed intermittent intraperitoneal treatment of RLV infected mice with AZT and with the liposomal AZT derivatives

Drug preparations and treatment schedules	Total dose		Spleen weight grams ± SD[a]	% Inhibition of splenomegaly
	mg/kg	mmol/kg		
Days 0, 3, 7, 11				
AZT in phosphate buffer	1000	3.74	ND[b]	ND[b]
N^4-hexadecyldC-AZT liposomes	2920	3.74	0.32 ± 0.15	73.4
N^4-palmitoyldC-AZT liposomes	2970	3.74	0.29 ± 0.10	82.3
Days 3, 7, 11				
AZT in phosphate buffer	1000	3.74	0.63 ± 0.18	28.0
N^4-hexadecyldC-AZT liposomes	2920	3.74	0.41 ± 0.11	62.0
N^4-palmitoyldC-AZT liposomes	2970	3.74	0.38 ± 0.15	66.1
Days 7, 11				
AZT in phosphate buffer	1000	3.74	0.77 ± 0.14	16.7
N^4-hexadecyldC-AZT liposomes	2920	3.74	0.46 ± 0.17	55.1
N^4-palmitoyldC-AZT liposomes	2970	3.74	0.62 ± 0.13	39.9
Controls infected	–	–	0.84 ± 0.09	0
Controls uninfected	–	–	0.10 ± 0.01	–

[a]SD, standard deviation, n = 7–10; evaluation on day 14.
[b]ND, not determined.

Although no significant difference ($P=0.55$; 0.78 and 0.14) was observed between the two AZT derivatives during these treatment schedules, both derivatives were significantly more effective than AZT in the treatment schedule of days 3, 7, and 11 with $P=0.046$ comparing AZT versus N^4-hexadecyldC-AZT and with $P=0.045$ with AZT vs. N^4-palmitoyldC-AZT. In the day 7 and 11 treatment schedule, a significant difference in inhibition of splenomegaly was only observed with the N^4-hexadecyldC-AZT derivative, with $P=0.03$, whereas the effects of AZT and N^4-palmitoyldC-AZT were not significantly different ($P=0.16$).

In Fig. 5, immunoblots of spleen extracts and sera from the different treatment regimens were analysed for viral antigens. In contrast to AZT (Fig. 5; lanes 3, 7, and 10) and independently from the different treatment schedules (days 1, 6, 11, and 16; days 3, 7, and 11; and days 7 and 11), spleen and serum samples from the animals treated with liposomal N^4-hexadecyldC-AZT (lanes 4, 8 and 11) or N^4-palmitoyldC-AZT (lanes 9 and 12) showed significantly reduced expression of the virus-specific antigens gp70 and p30.

Treatment of uninfected and RLV infected mice with high doses of drug-free control liposomes on days 1, 6, 11, and 16, corresponding to the highest dose of 12 g lipid/kg used with the AZT derivatives (see Fig. 4) revealed no antiviral effect. On the contrary, in both treated groups, an increase of the spleen weight of 50% and 66%, respectively, was observed. The effects of drug-free liposome administration

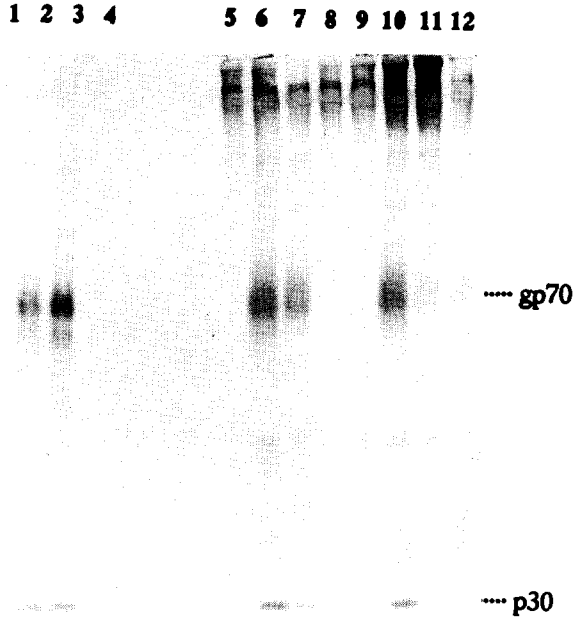

Fig. 5. Immunoblot of spleen (lanes 1–4) and serum (lanes 5–12) extracts. Spleen extracts from mice treated on days 1, 6, 11, 16 with N^4-hexadecyldC-AZT (lane 4) and free AZT (lane 3); lanes 1 and 2, uninfected and infected controls. Total AZT concentration was 1140 mg/kg (Fig. 4). Serum extracts from mice treated on days 3, 7, 11 with free AZT (lane 7), N^4-hexadecyldC-AZT (lane 8), N^4-palmitoyldC-AZT (lane 9) and on days 7 and 11 with free AZT (lane 10), N^4-hexadecyldC-AZT (lane 11), N^4-palmitoyldC-AZT (lane 12, Table 2). Lanes 5 and 6, serum samples from uninfected and infected controls. Molecular weight markers were run in parallel (not shown).

on organs of the MPS, particularly on the spleen was documented by Allen and collaborators (Allen et al., 1984) who reported a doubling of spleen weight in ICR mice after chronic liposome treatment. However, the effects on liver and spleen functions depend strongly on liposome composition and dosage, frequency of administration, vesicle size and population homogeneity (Allen 1988).

3.4. Haematological toxicity

The haematological parameters of uninfected mice treated i.p. and i.v. on days 1, 6, 11, and 16 with N^4-hexadecyldC-AZT or AZT dissolved in PB at total AZT concentrations of 380 mg/kg and 1140 mg/kg are summarised in Table 3. The effect of the lipophilic AZT derivative is generally comparable to that observed with free AZT. In the i.v. treated animals, AZT caused a stronger depression of the white blood cell (WBC) counts, whereas the drop of the WBC counts of the mice treated with N^4-hexadecyldC-AZT was less pronounced. In the i.p. treated groups, an increase of WBC counts was noted with N^4-hexadecyldC-AZT and with the lower dose of AZT. This increase of WBC was possibly caused by a local irritation of the peritoneal cavity, whereas AZT at 1140 mg/kg total dose might be toxic (see Fig. 4).

Table 3
Haematological toxicity of uninfected Balb/c mice treated on days 1, 6, 11 and 16 with N^4-hexadecyldC-AZT and AZT

Drug and application route	Total dose mg/kg	WBC[a] $10^3/\mu l$	Change %	RBC[b] $10^6/\mu l$	Change %	Hb[c] g/%	Change %
Intravenous treatment ($n=3$)							
N^4-hexadecyldC-AZT	1110	4.50 ± 1.2	−8.5	7.70 ± 1.34	−17	10.1 ± 2.5	−16
AZT	380	2.90 ± 0.1	−41	9.20 ± 0.06	− 0.4	11.9 ± 0.2	−0.8
Intraperitoneal treatment ($n=3$)							
N^4-hexadecyldC-AZT	1110	6.60 ± 1.0	+34	9.01 ± 0.82	−0.5	11.5 ± 0.9	−4
N^4-hexadecyldC-AZT	3330	5.30 ± 1.0	+8	7.99 ± 2.23	−13.5	10.6 ± 2.4	−12
AZT	380	5.10 ± 3.3	+4	8.20 ± 1.60	−11	10.7 ± 2.3	−11
AZT	1140	4.50 ± 1.3	−8.5	8.14 ± 1.66	−12	10.4 ± 2.0	−13
Controls untreated ($n=8$)	-	4.92 ± 2.1	-	9.24 ± 0.88	-	12.0 ± 1.5	-

[a]WBC, white blood cells; [b]RBC, red blood cells; [c]Hb, Haemoglobin.

With both treatments, the red blood cell (RBC) count dropped between 0.4 and 17%. However, no statistical differences at $P=0.05$ were found by the comparison of AZT and N^4-hexadecyldC-AZT with the values of uninfected mice in both the WBC and RBC counts (see Table 3). The changes observed in the WBC counts and haemoglobin concentration were comparable to those found by Ruprecht and coworkers after oral or i.p. treatment with AZT (Ruprecht et al., 1986).

4. Discussion

The lipophilic derivatives of AZT represent a new class of antiretroviral compounds with AZT as a protected active moiety of the molecules. In vitro, these derivatives inhibit cellular HIV-1 replication in a dose-dependent fashion, however, higher concentrations of these derivatives are required to obtain a similar antiviral effect as that obtained with free AZT. These higher concentrations may be explained by the slow uptake of the liposomes and micelles by the HeLa and H9 cells which are not known to have a high phagocytosing activity. Furthermore, in vitro the kinetics of conversion of the derivative molecules into active agents such as AZT, AZT-5′-monophosphate and the di- and triphosphorylated nucleosides may be slower than those of free AZT. Formation of other cellular metabolites of unknown structure and activity is conceivable and may further contribute to a delayed antiviral action. Hostetler et al. showed that similar lipophilic derivatives of AZT and 3′-deoxythymidine become associated with cell membranes which serve as an intracellular depot of the lipophilic derivatives (Hostetler et al., 1991; Hostetler et al., 1992).

To demonstrate the antiviral effects of the AZT derivatives in vivo, we chose the Rauscher leukemia virus (RLV) model. This model exhibits several advantages over

other murine virus models: (a) RLV infected mice develop an erythroleukemia followed by a massive splenomegaly within 2–3 weeks and; (b) the degree of splenomegaly is proportional to the virus titer (Brockbank et al., 1986). This phenomenon allows for a rapid and quantitative analysis of antiretroviral activity by determination of spleen weights (Ruprecht et al., 1986; Ruprecht et al., 1990b; Koch and Ruprecht 1992).

The superior activity of the AZT derivatives obtained in the in vivo experiments (Tables 1 and 2, Fig. 4) accentuate the importance of testing modified molecules with potential prodrug nature in animal models. In vitro, the full activation of the AZT derivatives which contain masked AZT or AZT-5′-monophosphate may not fully occur. Thus, in vitro test systems may not reflect true antiviral effects of such compounds and may therefore rather be used for pre-screening of compounds with unknown properties.

The liposomal AZT derivative preparations allow for treatment regimens with intermittent and delayed application schedules. Due to the fast metabolism and elimination of AZT (Doshi et al., 1989), the free drug is significantly less effective in these treatment regimens. Furthermore, the particulate nature of the liposomes may lead to higher drug levels in plasma and in virus-infected phagocytosing cells such as macrophages, monocytes and lymphocytes. Though the mechanism of action of the lipophilic AZT derivatives has not yet been elucidated, it is speculated that the drugs may act directly on the viral reverse transcriptase, or more likely, be converted to AZT or AZT-5′-monophosphate, and then further phosphorylated to AZT-triphosphate by cellular kinases. The cellular pharmacokinetics of these new derivatives are expected to be significantly different from those known for free AZT. The lipophilic properties of the AZT derivatives and the mechanisms involved in the cellular uptake of the liposomes may contribute to the superior antiviral effects of these compounds in vivo.

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