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Lipophilic 1-β-D-arabinofuranosyl cytosine derivatives in liposomal formulations for oral and parenteral antileukemic therapy in the murine L1210 leukemia model

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Abstract The N^4-alkylcytosine arabinoside derivative N^4-octadecyl-AraC (AraC-Ocd, NOAC) and the (1-octadecylglycero-3-phospho)-AraC (Ocd-Gro*P*-AraC, OPA) conjugate are new lipophilic derivatives of the cytostatic drug 1-β-D-arabinofuranosylcytosine (AraC) that produce high antileukemic effects in the L1210 murine leukemia model when administered orally or parenterally as liposomal formulations. Between 83% and 100% of the treated animals were cured after five consecutive daily oral drug applications with a total dose of 1 mmol/kg AraC-Ocd or Ocd-Gro*P*-AraC. Corresponding results were obtained after parenteral therapy on days 2 and 6 after tumor inoculation with five- to ten-fold lower concentrations of these two compounds. A comparable cytotoxic activity was found with the orally active AraC-5′-(*n*-stearyl phosphate). However, because of its strong hemolytic toxicity this derivative cannot be used for parenteral therapy. Another AraC conjugate, which was modified with two long-chain hydrocarbons, the (1-octadecylglycero-3-phospho)-N^4-hexadecyl-AraC was, probably because of poor oral bioavailability, only active when applied parenterally. The new lipophilic AraC derivatives AraC-Ocd and Ocd-Gro*P*-AraC are compounds with a high potential for the oral treatment of leukemias and possibly also of solid tumors.

Key words Lipophilic cytosine arabinoside derivatives · Liposomes · Oral therapy · L1210 leukemia

Abbreviations *AraC* 1-β-D-arabinofuranosylcytosine · *AraC-Ocd* N^4-octadecyl-1-β-D-arabinofuranosylcytosine · *Ocd-GroP-AraC* 5′-*O*-(1-octadecyl-*rac*-glycero-3-phospho)-1-β-D-arabinofuranosylcytosine · *OcdP-AraC* 1-β-D-arabinofuranosylcytosine-5′-(*n*-stearylphosphate) · *Ocd-GroP-AraC-Hxd* 5′-*O*-(1-octadecyl-*rac*-glycero-3-phospho)-N^4-hexadecyl-1-β-D-arabinofuranosylcytosine

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Introduction

The most important nucleoside analogue 1-β-D-arabinofuranosylcytosine (AraC), used for the parenteral chemotherapy of leukemias and lymphomas (Chabner 1990), has the two major clinical disadvantages of being ineffective after oral application and of fast kinetics of enzymatic deactivation to the metabolite arabinosyluracil (AraU) (Ho and Frei 1971). More recently, the potential usefulness of AraC derivatives containing lipophilic substituents as orally active drugs has been described. Ohno et al. (1986) reported a phase I study in patients suffering from leukemia and myelodysplastic syndromes where the derivative N^4-palmitoyl-AraC was given by the oral route. Kodama et al. (1989), Ohno et al. (1991) and Ueda et al. (1994) introduced 1-β-D-arabinofuranosylcytosine-5′-(*n*-stearylphosphate) (Ocd*P*-AraC, cytarabine ocfosfate, YNK01) as an orally active derivative of AraC. Koga et al. (1995) demonstrated the effectiveness of oral cytarabine ocfosfate against human colorectal adenocarcinoma xenografts with better efficacies than AraC and other antitumor drugs.

In previous studies, we reported on the excellent antitumor activity of liposomal formulations of the N^4-alkyl-AraC derivatives N^4-hexadecyl-AraC, NHAC) and N^4-octadecyl-1-β-D-arabinofuranosylcytosine (AraC-Ocd, NOAC). These new compounds were found to be extremely stable against deamination to AraU and of high antitumor activity (Schwendener and Schott 1992; Schott et al. 1994; Schwendener et al. 1995). Pharmacological studies revealed that this new

class of lipophilic AraC derivatives exert their cytotoxic action by mechanisms that are different from those of the parent compound AraC (Horber et al. 1995a–d). Another group of lipophilic AraC derivatives was obtained by linking AraC or N^4-alkylated AraC derivatives to phospholipids containing long-chain hydrocarbon ethers. Using parenteral application schedules, these phospholipid-AraC conjugates were shown to exert antileukemic effects that are comparable to those of the N^4-alkylated AraC derivatives (Schott and Schwendener 1996).

In the following it will be shown that excellent antileukemic effects of AraC conjugates, prepared by the coupling of one lipophilic hydrocarbon substituent via an ether linkage at the 5′-glycerophosphate, can also be obtained by oral application.

Materials and methods

Chemicals and lipids

The following AraC derivatives were synthesized in our laboratory according to published methods: the N^4-alkyl derivative N^4-octadecyl-1-β-D-arabinofuranosyl-cytosine (AraC-Ocd) (Schott et al. 1994) and the conjugates 5′-*O*-(1-octadecyl-*rac*-glycero-3-phospho)-1-β-D-arabinofuranosylcytosine (OPA) and 5′-*O*-(1-octadecyl-*rac*-glycero-3-phospho)-N^4-hexadecyl-1-β-D-arabinofuranosylcytosine (Ocd-Gro*P*-AraC-Hxd, OP-N^4-hexadecyl-AraC) (Schott and Schwendener 1996). 1-β-D-Arabinofuranosylcytosine-5′-(*n*-stearylphosphate) (Ocd*P*-AraC) was prepared analogously to 1-β-D-arabinofuranosylcytosine 5′-(*n*-hexadecylphosphate) (Rosowsky et al. 1982). Soy phosphatidylcholine was obtained from L. Meyer, Hamburg, Germany. Cholesterol, recrystallized from methanol, was from Fluka AG, Buchs, Switzerland. DL-α-Tocopherol, sodium cholate and all analytical-grade buffer salts were from Merck, Darmstadt, Germany. All other chemicals were of analytical grade (Merck and Fluka). AraC (Sigma, St. Louis, Mo.) was freshly dissolved in phosphate buffer at 40 mg/ml.

Liposome preparation

Small unilamellar liposomes were prepared by detergent dialysis of mixed lipid/AraC-conjugate/sodium-cholate micelles as described by Rubas et al. (1986). The lipids, sodium cholate as detergent and the lipophilic conjugates were dissolved in methylene-chloride/methanol (1:1, v/v) and the solvents were thoroughly removed in a rotatory evaporator at 37°C. The residue was solubilized in phosphate buffer (67 mM, pH 7.4) to give a mixed drug/lipid micelle solution, which was composed of 40 mg soy phosphatidylcholine, 4 mg cholesterol, 0.25 mg DL-α-tocopherol, 5 mg conjugate and 55 mg sodium cholate/ml. The micellar solutions were dialyzed against 10 l phosphate buffer during 20 h in a Lipoprep GD-1 instrument (Dianorm, Munich, Germany). The temperature of the dialysate was 40°C during the first 1–2 h of dialysis and, for the remaining time, dialysis was continued at room temperature. After dialysis, the liposome preparations were filtered through 0.45 µm sterile filters (Gelman, Ann Arbor, Mich., USA) and stored at 4°C. For the oral treatment experiments the liposomes were concentrated in a Amicon ultrafiltration cell (Amicon Corp., Lexington, Mass.) using Diaflo YM-100 membranes with a cut-off of 100000 M_r to obtain drug concentrations of 30–40 mg/ml.

Antitumor activity against L1210 leukemia

The antitumor activity of the liposomal drug preparations was evaluated with the murine L1210 leukemia model in BDF1 mice (Schwendener and Schott 1992). The L1210 cells (1×10^5 in 0.1 ml) from donor ascites were injected intravenously on day 0. Groups of five to seven BDF1 mice (18–25 g body weight, females; Charles River, Sulzfeld, Germany) were kept in Makrolon cages and fed with water and solid diet at libitum. Before each oral treatment the mice were fasted overnight. The drugs were applied by the use of a stomach catheter. A volume of 0.5 ml liposomes containing the AraC derivatives was given as five consecutive applications on days 1–5 after tumor cell injection. Control groups received AraC in 0.5 ml phosphate buffer or were left untreated. Correspondingly, the liposomal drugs were also applied by intravenous or intraperitoneal injections (Table 1). The increase of lifespan of treated animals (T) was evaluated by recording the mean survival time as compared to that of the untreated controls (C), expressed as T/C (%). The animals were observed daily until death or for 60 days. Animals surviving for 60 days were included into the calculation and considered as being cured.

In vitro hemolytic activity of the AraC conjugates

Liposome preparations of the derivatives AraC-Ocd, Ocd-Gro*P*-AraC and Ocd*P*-AraC were incubated at different concentrations (0–4 mM) with freshly collected human blood from healthy donors for 60 min at 37°C. Aliquots of the supernatants obtained after centrifugation (1500 *g*, 30 min) were diluted 1:100 in 0.9% NaCl and the concentration of hemoglobin was determined in a spectrophotometer by calculating the difference between the absorbances at 577 nm and 561 nm. Total hemolysis (100%) was obtained by incubation of erythrocytes in water containing 0.02% Triton X-100 (Sigma, St. Louis, Mo.) at a 1:1 (v/v) ratio.

Results

The structures of the AraC derivatives that were tested in the L1210 murine leukemia model are shown in Fig. 1. The antileukemic effects of these compounds after oral application are summarized in Table 1. The lipophilic N^4-alkyl-AraC derivative AraC-Ocd as well as the amphiphilic conjugate Ocd-Gro*P*-AraC are highly effective against L1210 leukemia when administered by either oral or parenteral application.

The antileukemic effects after oral application of the three AraC derivatives are comparable to those of Ocd*P*-AraC. A significant difference, however, is that the derivatives AraC-Ocd and Ocd-Gro*P*-AraC were found to be active after both oral and parenteral application, whereas Ocd*P*-AraC cannot be used for parenteral therapy because of its strong hemolytic activity, which is shown in Fig. 2. In the therapeutically active concentration range the derivatives AraC-Ocd and Ocd-Gro*P*-AraC have practically no hemolytic activity.

Surprisingly, the (1-octadecylglycero-3-phospho)-N^4-hexadecyl-AraC conjugate (Ocd-Gro*P*-AraC-Hxd) was not active after oral application, whereas all animals treated via the intraperitoneal route with a total dose of 0.2 mmol/kg were cured (Table 1). All orally active AraC derivatives exert a significantly higher

antileukemic activity as compared to AraC which, after oral application of 2 mmol/kg, produced a T/C value of 221% without any animals surviving. The relatively high T/C values obtained with all drugs and treatments may be due to the L1210 cells, which may have lost some of their original aggressiveness.

Discussion

The introduction of lipophilic residues into AraC resulted in lipophilic or amphiphilic AraC derivatives that possess a markedly increased antileukemic activity. To achieve an optimal bioavailability after oral application the AraC derivatives have to be given as liposome formulations. Although the liposomes are likely to be transformed into micellar structures upon interaction with the bile acids in the small intestine, such mixed drug/phospholipid/cholesterol micellar structures may confer a favorable drug uptake through the intestines. An alternative oral preparation, such as a suspension of drug crystals in peanut oil, proved to be less effective (Schwendener et al. 1996). However, in order to obtain oral formulations of the active AraC derivatives with optimal resorption characteristics further studies have to be carried out.

The derivatives AraC-Ocd and Ocd-Gro*P*-AraC were shown to have practically no hemolytic activity (Fig. 2). In contrast to Ocd*P*-AraC, which can only be applied by

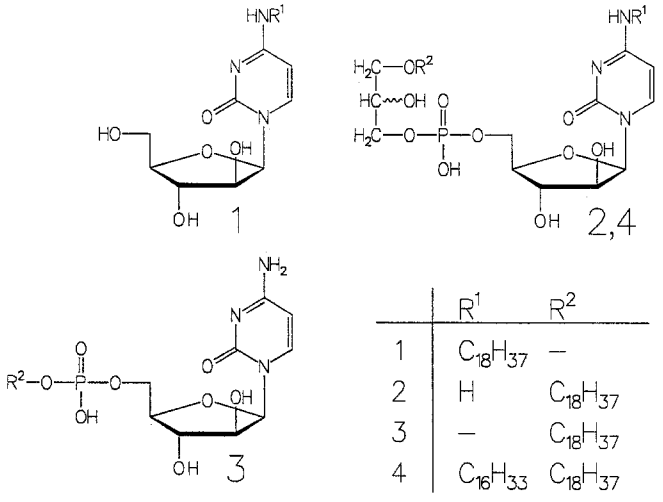

Fig. 1 Structure formulas of the N^4-alkyl-AraC derivative N^4-octadecyl-1-β-D-arabinofuranosylcytosine *1*, AraC-Ocd, 5′-*O*-(1-octadecyl-*rac*-glycero-3-phospho)-1-β-D-arabinofuranosylcytosine *2*, Ocd-Gro*P*-AraC, 1-β-D-arabinofuranosylcytosine-5′-(*n*-stearylphosphate) (*3*, Ocd*P*-AraC) and 5′-*O*-(1-octadecyl-*rac*-glycero-3-phospho)-N^4-hexadecyl-1-β-D-arabinofuranosylcytosine (*4*, Ocd-Gro*P*-AraC-Hxd)

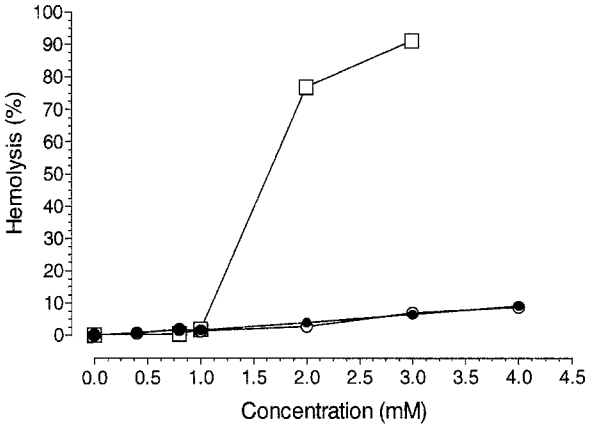

Fig. 2 In vitro hemolytic activity of the AraC derivatives. The drugs were incubated with human blood and the hemolytic activity was determined in the supernatant of the samples after centrifugation (1500 *g*, 30 min). ● AraC-Ocd, ○ Ocd-Gro*P*-AraC, □ Ocd*P*-AraC

Table 1 Comparison of oral (p.o.) and parenteral (i.p., i.v.) treatment of L1210 murine leukemia with liposomal N^4-octadecyl-1-β-D-arabinofuranosylcytosine (AraC-Ocd), 5′-*O*-(1-octadecyl-*rac*-glycero-3-phospho)-1-β-D-arabinofuranosylcytosine (Ocd-Gro*P*-AraC), AraC-5′-(*n*-stearylphosphate) (Ocd*P*-AraC) and 5′-*O*-(1-octadecyl-*rac*-glycero-3-phospho)-*N*-hexadecyl-AraC (Ocd-Gro*P*-AraC-Hxd). On day 0, 10^5 L1210 cells were injected intravenously into BDF1 mice. The total dose was given orally on 5 consecutive days or i.p. and i.v. on days 2 and 6. The increase of lifespan, T/C (%) was calculated including the 60-day survivors

Drug and application route		Total dose		Survival (days), mean ± SD	T/C[4] (%)	Survivors (60) days	
		(mmol/kg)	(mg/kg)			(%)	(no.)
AraC-Ocd	p.o.	1	496	54 ± 15	673	83	5/6
	i.v.	0.1	50	> 60	857	100	6/6
Ocd-Gro*P*-AraC	p.o.	1	650	54 ± 15.5	671	83	5/6
Ocd-Gro*P*-AraC-Hxd	i.p.	0.1	65	> 60	750	100	6/6
	p.o.	1	863	8 ± 0.6	100	0	0/6
Ocd*P*-AraC	i.p.	0.2	173	> 60	750	100	6/6
	p.o.	1	574	> 60	750	100	6/6
	i.v.[a]	—	—	—	—	—	—
AraC	p.o.	2	486	18 ± 17	221	0	0/5
Controls	—	—	—	8	100	0	0/7

[a] Parenteral treatment was not possible because of the hemolytic toxicity (Fig. 2)

the oral application route (Table 1 and Schleyer et al. 1995), these new derivatives exert their antitumor effects after parenteral treatment without causing hemolysis. Thus, the absence of amphiphilic properties of these derivatives represents a prerequisite for safe parenteral drug application. The two derivatives Ocd-Gro*P*-AraC and Ocd*P*-AraC differ only in an additional glycerol backbone, which is inserted between the 5′-phosphate group and the octadecyl chain in Ocd-Gro*P*-AraC. Surprisingly, this difference provides Ocd-Gro*P*-AraC with properties that are distinctly different from those of Ocd*P*-AraC. Ocd-Gro*P*-AraC has no hemolytic toxicity and has an excellent antileukemic activity, which is comparable to that of AraC-Ocd.

The chemical modification of AraC to lipophilic derivatives can be accomplished in different ways. Derivatives that are obtained by alkylation of the N^4 position of AraC with long-chain hydrocarbons (e.g. AraC-Ocd) have an oral antileukemic activity that is comparable to that of the conjugates where AraC is linked to an ether lipid, as in Ocd-Gro*P*-AraC.

The additional alkylation of Ocd-Gro*P*-AraC at the N^4-position of the cytosine moiety with long-chain hydrocarbons yields derivatives that are completely inactive after oral therapy. An example of such a derivative containing two alkyl modifications is Ocd-Gro*P*-AraC-Hxd; all animals treated with this conjugate by intraperitoneal application were cured, but oral treatment of L1210 leukemic mice was ineffective (Table 1). The lack of oral activity of this derivative is probably due to the poor bioavailability of this highly lipophilic compound.

On the basis of these results, the more complicated synthesis of the AraC conjugates Ocd-Gro*P*-AraC and Ocd-Gro*P*-AraC-Hxd does not justify their use as antileukemic compounds when they are compared to the more effective derivative AraC-Ocd, which is simpler and less costly to synthesize. However, the possibility cannot be ruled out that, because of their different chemical structure, these conjugates might possess different tumor specificities. Therefore, further investigations are necessary to evaluate their toxicity, pharmacokinetic profiles and cytotoxic activity against solid tumors after oral treatment.

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