SELECTIVE INHIBITION OF ECTROMELIA VIRUS DNA SYNTHESIS IN HEPATOCYTES BY ADENINE-9-β-D-ARABINOFURANOSIDE (ARA-A) AND ADENINE-9-β-D-ARABINOFURANOSIDE 5'-MONOPHOSPHATE (ARA-AMP) CONJUGATED TO ASIALOFETUIN

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1. Introduction

Some glycoproteins, such as fetuin and orosomucoid, after sialic acid removal and consequent exposure of galactose residues are selectively taken up by hepatocytes where they are hydrolyzed in lysosomes [1–3]. These desialylated glycoproteins have been used as carriers for introducing proteins [4] and liposomes [5] in parenchymal liver cells. We have coupled trifluorothymidine (F_3T) to asialofetuin (AF) [6] in order to selectively concentrate an inhibitor of DNA synthesis into hepatocytes. The resulting conjugate (F_3T–AF) was employed as an approach to the lysosomotropic [7,8] treatment of hepatitis caused by deoxyriboviruses [6,9,10]. F_3T coupled to AF caused an inhibition of viral DNA synthesis in liver of Ectromelia virus-infected mice which was >3-times greater than that produced by the free drug. On the contrary inhibition of DNA synthesis in bone marrow was the same when F_3T was administered either coupled to AF or as a free drug [6]. The inhibition of DNA synthesis caused by F_3T–AF in bone marrow appears to be due to F_3T released from the conjugate inside the hepatocytes and escaped from these cells into the blood [6].

Contrary to other inhibitors of DNA synthesis, such as cytosine-1-β-D-arabinofuranoside and adenine-9-β-D-arabinofuranoside (ara-A), F_3T is not used as a systemic antiviral drug in man because of its toxicity. ara-A has been employed in the treatment of human chronic hepatitis B (HB) infection [11–13]. It inhibits HB virus replication [11–13] but produces side effects, mainly gastrointestinal symptoms [11,14] which are most likely due to its inhibitory effect on the dividing cells of gastric and intestinal mucosa. These side effects could be avoided or reduced by concentrating ara-A into hepatocytes. We report here the conjugation of ara-A and ara-A 5'-monophosphate ara-A and ara-AMP were administered to Ectromelia activity as ara-A [15]. The conjugates as well as free ara-A and ara-AMP were administered to Extromelia virus-infected mice, during the phase of virus replication in hepatocytes [6], and their effect on virus DNA synthesis in liver and on cell DNA synthesis in the intestine was determined.

2. Materials and methods

2.1. *ara-A and ara–AMP conjugates*

Fetuin (Sigma type III) was desialylated by neuraminidase according to [16].

The conjugate of ara-A (ara-A–glut-AF) was obtained by coupling ara-A–glutarate to AF via its hydroxysuccinimide ester. 500 mg ara-A (1.87 mmol) (Serva, D-69 Heidelberg: dried at 80°C overnight over P_4O_{10} in vacuo) was dissolved in 10 ml pyridine, and glutaric anhydride (259 mg, 2.27 mmol) was added over 2 h with stirring, at 80°C and left overnight at the same temperature. The solvent was evaporated, the residue was dissolved in 1.5% aqueous acetic acid and chromatographed on a Sephadex LH-20 column (200 × 3 cm). Fractions 15–28 (each fraction 20 ml) were evaporated and rechromato-

graphed on the same column; fractions 47–56 (10 ml each) contained ara-A glutarate and were lyophilized (120 mg) (high-voltage paper electrophoresis at pH 6.5 showed the presence of a main band which migrated to the anode). The product (120 mg, 0.315 mmol) was dissolved in 5 ml dimethylformamide and *N*-hydroxysuccinimide (43 mg, 0.37 mmol) and dicyclohexylcarbodimide (77 mg, 0.37 mmol) were added gradually at 4°C. After 72 h at the same temperature the precipitate was filtered off and the solution added to 200 mg AF dissolved in 20 ml water, maintaining 4°C and pH 6–7; after the addition (4 h), the solution was left overnight at the same temperature, then dialyzed and lyophilized. The product was dissolved in 10 ml 0.05% ammonium bicarbonate solution, and chromatographed through a Sephadex G-75 column (100 × 2 cm) and eluted with the same solvent, and lyophilized. Two fractions (A,B) were collected (150 and 21 mg), with a 80% protein content as determined by the method in [17] and 8 mol ara-A/mol AF (UV spectrophotometry). Sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis showed that fraction A contained polymers of AF whereas fraction B contained the monomeric form of AF. ara-AMP was coupled to AF (ara-AMP–AF) by a water-soluble carbodiimide as in [18]. 60 mg 1-ethyl-3-(dimethyl-aminopropyl)-carbodiimide (ECDI) (Fluka) dissolved in 200 μl H_2O was added to 60 mg ara-AMP dissolved in 1 ml H_2O by addition of $NaHCO_3$ (pH 5). After 10 min at 25°C, 30 mg AF dissolved in 300 μl H_2O (adjusted to pH 7 with NaOH), was added with shaking. The mixture was incubated at 25°C for 24 h, then chromatographed on a Sephadex G-100 column (1.6 × 90 cm) equilibrated and eluted with 0.15 M NaCl. The conjugated material emerged in two peaks. SDS–polyacrylamide gel electrophoresis showed that the first peak contained polymers of AF, whereas the second peak contained the monomeric form of AF. Fractions corresponding to the second peak were pooled, dialyzed against water and lyophilized. The yield in monomeric form of the conjugate (ara-AMP–AF) was 10 mg. Three different preparations were made by this procedure (I,II,III). The molar ratio of ara-AMP:AF in the conjugates ranged from 3.5–4 as determined spectrophotometrically. In a separate experiment AF was maintained in the presence of ara-AMP with the same procedure used for the preparation of ara-AMP–AF, except that ECDI was omitted. After incubation at 25°C for 24 h ara-AMP was removed by gel filtration. No ara-AMP was found adsorbed onto AF as assessed by spectrophotometric measurement, indicating that in the conjugates ara-AMP is linked to AF by a covalent bond. Probably conjugation takes place mostly by formation of an amide bond between the ϵ-NH_2 group of lysine in AF and the phosphate group of ara-AMP, as shown in an analogous reaction using carbodiimides for coupling thymidilic acid to albumin [18].

2.2. *Blood clearance of labeled AF*

The blood clearance of ^{14}C-labelled AF in mice in the presence of cold non-conjugated AF, ara-A–glut-AF and ara-AMP–AF was determined as in [6].

2.3. *DNA synthesis in liver and intestine of Ectromelia virus-infected mice*

Ectromelia virus (Hampstead mouse strain) was grown in L-929 cells and purified according to [19,20]. In each experiment 14 Swiss male mice (28–31 g) were injected intravenously (i.v.) with Ectromelia virus at the multiplicity of 2×10^5 p.f.u./g body wt, corresponding to 300-times the LD_{50}. 44 h after infection, i.e., during the phase of virus replication in hepatocytes [6], mice were divided in 2 groups of 7 animals each. Animals of one group were used as controls. Animals of the other group were inoculated intraperitoneally (i.p.) with 2′-deoxycoformycin (an inhibitor of ara-A deamination [21,22]) (2 μg/g body wt) and 45 min later received an i.v. injection of the antiviral compound to be tested. After 45 min animals of treated and control groups were injected i.p. with [*methyl*-^{3}H]thymidine (spec. act. 24 Ci/mmol) at 30 μCi/animal. Thirty min after [*methyl*-^{3}H]thymidine administration mice were killed and liver as well as a tract of intestine (4 cm long starting from pylorus) were rapidly removed. DNA was extracted from these tissues according to [23] its radioactivity was counted and concentration was measured according to [24].

Administration of 2′-deoxycoformycin was necessary because ara-A is >100-times less active in rodents than in primates, due to its rapid inactivation mostly by oxidation at C-2 position and in part by deamination [25]. In fact, doses of ara-A and ara-AMP alone up to 5 μg (19 nmol) and 10 μg (29 nmol)/g body wt, respectively, did not produce any significant inhibition of DNA synthesis in liver and intestine of Ectromelia virus-infected mice. 2′-Deoxycoformicin

administered alone to Ectromelia virus-infected mice, was found to be ineffective on DNA synthesis in liver and intestine.

3. Results and discussion

Conjugation did not change the capacity of AF to interact with the specific receptors on the surface of hepatocytes. Indeed, the clearance of ^{14}C-labelled AF from the blood of mice was competitively inhibited to the same extent by the conjugates and by an equal amount of non conjugated AF (table 1).

Table 2 shows that free ara-A and ara-AMP produced a greater inhibition of DNA synthesis in intestine than in liver. On the contrary ara-A–glut-AF and ara-AMP–AF inhibited DNA synthesis in liver without producing any significant inhibition in the intestine. Doses of conjugated ara-A and ara-AMP 2–4-times lower than those of the free drugs were required to produce a comparable inhibition of DNA synthesis in liver. These results indicate that, after injection of the conjugates, ara-A and ara-AMP were concentrated in the active form into hepatocytes. In the case of ara-AMP–AF it cannot be excluded that the ester bond linking phosphate to ara-A was not broken down in hepatocyte lysosomes so that the drug released free from the conjugate was ara-A and not ara-AMP.

Table 1
Effect of cold non-conjugated AF and conjugates on plasma clearance of ^{14}C-labelled AF

Compounds injected with ^{14}C-labelled AF	dpm/ml plasma
None	54 900
AF	112 000
ara-A–glut-AF(A)	98 950
ara-A–glut-AF(B)	120 260
ara-AMP–AF(I)	123 050

Mice were injected i.v. with 2 μg/g body wt of ^{14}C-labelled AF (5×10^6 dpm/mg). Cold AF or conjugates were administered i.v. simultaneously with [^{14}C] AF at 20 μg/g body wt. After 5 min animals were killed and the radioactivity of plasma was measured. Each entry represents the mean value of results from 2 animals

The rate of uptake of AF by hepatocytes is limited (0.2 μg . g body wt^{-1} . min^{-1} in the rat [26]). This may be the reason why the conjugates did not produce an inhibition of DNA synthesis in liver >50–59%. A greater inhibition might be obtained in animals, such as man, where ara-A is more effective than in rodents, even in the absence of 2′-deoxycoformicin [25].

Table 2
Inhibition of DNA synthesis in liver and intestine of Ectromelia virus-infected mice after injection of free or conjugated ara-A and ara-AMP

Exp. no.	Compound injected	ara-A administered (nmol/g body wt)	Inhibition of DNA synthesis (%)	
			Liver	Intestine
1	ara-A	13.1	42 $P < 0.001$[b]	60 $P < 0.001$
2	ara-A	18.7	58 $P < 0.005$	68 $P < 0.001$
3	ara-AMP	8.6	57 $P < 0.005$	60 $P < 0.001$
4	ara-AMP	17.2	66 $P < 0.001$	77 $P < 0.001$
5	ara-AMP	34.5	77 $P < 0.001$	89 $P < 0.001$
6	ara-A–glut-AF (A)	5.6 (33)[a]	38 $P < 0.02$	15 n.s.
7	ara-A–glut-AF (B)	1.9 (11)	26 $P < 0.05$	8 n.s.
8	ara-A–glut-AF (B)	5.6 (33)	43 $P < 0.01$	16 n.s.
9	ara-AMP–AF (I)	1.8 (22)	38 $P < 0.005$	0
10	ara-AMP–AF (II)	1.1 (15)	44 $P < 0.001$	0
11	ara-AMP–AF (II)	2.2 (30)	59 $P < 0.001$	19 n.s.
12	ara-AMP–AF (III)	2.8 (42)	53 $P < 0.005$	13 n.s.

[a] In parentheses the amount of conjugate injected (in μg/g body wt)

[b] Results were statistically evaluated by means of Student's t-test. The difference was considered not statistically significant (n.s.) for $P < 0.05$

Experimental details are given in section 2.3

For the same amount of bound ara-A, ara-AMP-AF seemed more effective than ara-A–glut-AF. This could depend on the different bonds between the drugs and the carrier affecting drug release in lysosomes.

The use of AF conjugates of ara-A or ara-AMP in human chronic hepatitis B should reduce the side effects occuring after the use of free ara-A [11,14]. These and similar conjugates could also be useful in the treatment of hepatomas, provided that neoplastic hepatocytes maintain the receptors for AF. Concentration of the inhibitor of DNA synthesis in liver cells should kill neoplastic proliferating hepatocytes without damaging non-dividing normal hepatocytes.

A drawback in the clinical use of these conjugates is the possibility that antibodies could be produced against them. This risk might be reduced by administering conjugates prepared with galactosylated homologous serum albumin. Indeed homologous albumin is not immunogenic. In addition, it has been found that albumin after galactosylation is selectively taken up by hepatocytes [27,28].

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