HEPATOCYTE TARGETING OF ADENINE-9-β-D-ARABINOFURANOSIDE 5'-MONOPHOSPHATE (ara-AMP) COUPLED TO LACTOSAMINATED ALBUMIN

L. FIUME, C. BUSI, A. MATTIOLI, P. G. BALBONI* and G. BARBANTI-BRODANO*
Istituto di Patologia generale, Via San Giacomo 14, 40126 Bologna and **Istituto di Microbiologia, Via Luigi Borsari 46, 44100 Ferrara, Italy*

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1. Introduction

The administration of inhibitors of DNA synthesis as antiviral agents is limited by their toxicity to proliferating cells. Therefore the treatment of diseases caused by deoxyriboviruses growing in non-dividing cells would be improved by selectively concentrating these drugs in the infected cells [1–4]. Concentration of trifluorothymidine [2], adenine-9-β-D-arabinofuranoside (ara-A) and ara-A monophosphate (ara-AMP) [3] inside hepatocytes has been achieved in mice with *Ectromelia* virus-hepatitis by injecting these drugs coupled to asialofetuin (AF). Fetuin, a glycoprotein from fetal calf serum, when administered to mammals in a desialylated form, binds to a receptor for galactosyl-terminating glycoproteins present only on hepatocytes and consequently is selectively internalized in these cells where it is digested in lysosomes [5–7]. It has been suggested [8,9] that a protein may be made to penetrate into a cell which it does not normally enter by itself, by coupling it with a smaller molecule for which a specific binding site exists on the cell membrane. In agreement with this hypothesis it was observed that proteins, after coupling to the asialoglycopeptide of fetuin [10] or simply to lactose [11,12], enter into parenchymal liver cells.

We have found that lactosaminated serum albumin (L-SA) can substitute AF as a hepatotropic carrier of ara-AMP. L-SA has the advantage over AF of being easily obtained in the amounts required for clinical purposes. Moreover the use of homologous L-SA might reduce the risk of an immune response against the conjugate. ara-AMP displays the same antiviral activity as ara-A [13]; both drugs must be converted to ara-A triphosphate in order to inhibit DNA synthesis [14,15]. ara-AMP was chosen because it can be easily bound to proteins by means of water-soluble carbodiimides [3].

2. Materials and methods

2.1. *L-SA–ara-AMP conjugates*

Lactose was coupled to ϵ-NH_2 of lysine residues of rabbit (RSA) or human (HSA) serum albumin by reductive amination with cyanoborhydride [16,17]. The albumin concentration was determined as in [18]. The amount of sugar conjugated to protein was established by the phenol/sulfuric acid method [19] calibrated against galactose. In different preparations of L-SA increasing amounts of lactose were coupled as a function of time of reaction [11,17]. ara-AMP was conjugated to L-SA by the use of 1-ethyl-3-(dimethyl-aminopropyl)-carbodiimide, following the procedure used to bind this drug to AF [3]. Conjugation takes place probably by formation of an amide bond between the ϵ-NH_2 group of lysine in the protein and the phosphate group of ara-AMP [20]. After gel chromatography on Sephadex G-100 [3] the fractions corresponding to the monomeric form of the conjugate were pooled, dialyzed against water and lyophilized. One L-RSA–ara-AMP and 3 L-HSA–ara-AMP conjugates were prepared. The molar ratio ara-AMP/albumin was determined spectrophotometrically. During ara-AMP conjugation the amount of galactosyl residues attached to albumin was found to decrease. The molar ratio sugar/albumin was therefore measured again in L-SA–ara-AMP conjugates taking into consideration the contribution given by arabinose in the phenol/sulphur reaction.

2.2. *[methyl-^{3}H]Thymidine incorporation into DNA in liver, intestine and bone marrow of Ectromelia virus-infected mice*

In each experiment purified [21,22] *Ectromelia* virus (Hampstead mouse strain) was injected intravenously (i.v.) into 14 Swiss female mice (26–28 g) at the multiplicity of 2×10^5 p.f.u./g body wt. After 44.5 h 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 9-erithro-(2-hydroxyl-3-nonyl)-adenine (EHNA) (an inhibitor of ara-A deamination [23])(3 μg/g body wt) and 15 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 (25 Ci/mmol) at 30 μCi/animal. After 30 min mice were killed and liver, a tract of intestine (4 cm long starting from pylorus) and bone marrow from femurs were rapidly removed. DNA was extracted according to [24], the radioactivity was counted and the concentration measured as in [25]. Administration of EHNA was necessary [3] because ara-A is >100-times less active in rodents than in primates, due to its more rapid metabolism [26]. EHNA administered alone to *Ectromelia* virus-infected mice, did not affect thymidine incorporation in liver, intestine or bone marrow.

3. Results and discussion

L-SA–ara-AMP conjugates interact with the hepatic receptor for asialoglycoproteins; in fact, as shown in table 1, they competitively inhibited the blood clearance of [^{14}C]AF in mice.

Free ara-A and ara-AMP as well as coupled ara-AMP were administered to mice 44.75 h after infection with *Ectromelia* virus and their effect on thymidine incorporation into DNA in liver, intestine and bone marrow was determined. After 46 h *Ectromelia* virus infection the newly synthesized liver DNA hybridizes with viral DNA (table 2); electron microscopic observations (M. Derenzini, unpublished) demonstrated that at this time the infection is widespread to the hepatic parenchyma in agreement with [27] and that the number and size of viral factories as well as the number of viral particles they contain are several times higher in parenchymal than in sinusoidal cells. This indicates that in liver, in our experimental conditions, most of the virus DNA synthesis occurred

Table 1
Effect of L-SA–ara-AMP conjugates on plasma clearance of ^{14}C-labelled AF

Exp. no.	Compounds injected with ^{14}C-labelled AF	dpm/ml plasma
1	None	1175 ± 420
2	AF	2320 ± 156
3	$L_{25.5}$-RSA–ara-$AMP_{4.0}$	2431 + 248
4	$L_{21.6}$-HSA–ara-$AMP_{7.5}$	1797 ± 313
5	$L_{21.6}$-HSA–ara-$AMP_{7.5}$	2201 ± 293
6	$L_{21.2}$-HSA–ara-$AMP_{10.9}$	1953 ± 2
7	$L_{23.2}$-HSA–ara-$AMP_{11.2}$	2224 ± 20

Swiss female mice (26–28 g) were injected i.v. with 2 μg/g body wt of ^{14}C-labelled AF (7×10^5 dpm/mg) prepared according to [36]. Cold AF or conjugates were administered simultaneously with [^{14}C]AF at 20 μg/g body wt except in expt 5 in which the conjugate was injected at 33 μg/g (an amount equimolecular to 20 μg AF). After 5 min animals were killed and the radioactivity of plasma was measured. Each entry represents the mean value of results from 2 animals. Number subscript to L and ara-AMP represents the molar ratios of sugar/albumin and ara-AMP/albumin, respectively

Table 2
Hybridization to *Ectromelia* virus DNA of liver [^{3}H]DNA from normal and *Ectromelia* virus-infected mice

DNA immobilized on filters	Liver [^{3}H]DNA added to filters from	cpm retained on filters
Ectromelia virus	Normal mice	31
Ectromelia virus	*Ectromelia* virus -infected mice	522
Salmon sperm	Normal mice	29
Salmon sperm	*Ectromelia* virus -infected mice	20

[*methyl*-^{3}H]Thymidine (47.5 Ci/mmol) was injected i.p. (100 μCi/animal) to normal and *Ectromelia* virus-infected mice (46 h post infection). After 30 min mice were killed and the liver was removed. DNA was extracted from purified *Ectromelia* virus and from liver as in [37]. Filter hybridization was performed as in [38]. *Ectromelia* virus or salmon sperm DNA (5 μg) were immobilized on each filter and 8×10^3 cpm of labelled liver DNA were added. Each result represents the mean value from 2 filters

Table 3
Inhibition of thymidine incorporation into DNA in liver, intestine and bone marrow of *Ectromelia* virus-infected mice after injection of free ara-A, ara-AMP and conjugated ara-AMP

Exp. no.	Compound injected	ara-A administered (nmol/g body wt)	Inhibition of thymidine incorporation		
			Liver	Intestine	Bone marrow
1	ara-A	9.4	45(S)[c]	44(S)	0
2	ara-A	13.0	50(S)	55(S)	25(S)
3	ara-AMP	34.5	50(S)	61(S)	43(S)
4	$L_{25.5}$-RSA–ara-$AMP_{4.0}$	1.0(20.7)[a]	30(S)	0	n.d.[b]
5	$L_{25.5}$-RSA–ara-$AMP_{4.0}$	2.0(41.5)	53(S)	23(S)	n.d.[b]
6	$L_{21.6}$-HSA–ara-$AMP_{7.5}$	1.6(16.6)	21(NS)[c]	0	0
7	$L_{21.6}$-HSA–ara-$AMP_{7.5}$	4.2(43.5)	43(S)	0	0
8	$L_{21.2}$-HSA–ara-$AMP_{10.9}$	4.9(35)	50(S)	19(NS)	2(NS)
9	$L_{23.2}$-HSA–ara-$AMP_{11.2}$	4.0(28.2)	48(S)	0	0
10	$L_{23.2}$-HSA–ara-$AMP_{11.2}$	4.8(35)	58(S)	18(NS)	0

[a] In parenthesis the amount of conjugate injected (in μg/g body wt); [b] n.d., not done
[c] Results were statistically evaluated by means of Student's *t*-test. The difference was considered statistically significant (S) or not significant (NS) for $P < \text{or} > 0.05$, respectively

in hepatocytes. As shown in table 3, and in agreement with [3], free ara-A and ara-AMP inhibited DNA synthesis to the same extent in liver and intestine; they produced a smaller inhibition in bone marrow. On the contrary inhibition of DNA synthesis caused by ara-AMP coupled to L-SA was higher in liver than in intestine where in some experiments thymidine incorporation was completely unaffected. Conjugated ara-AMP did not interfere with DNA synthesis in bone marrow. Doses of conjugated ara-AMP lower than those of free drugs were required to cause a comparable inhibition of DNA synthesis in liver. These results indicate that, after injection of the conjugates, ara-AMP is concentrated in a pharmacologically active form into hepatocytes. Since the ester bond linking phosphate to ara-A might be broken down in hepatocyte lysosomes, it can not be excluded that the drug released free from the conjugates was ara-A and not ara-AMP.

Viral hepatitis B occurs endemically in all parts of the world and is a major medical problem because of the impact that it has on blood transfusion services and its association with chronic liver disease and with primary liver cancer [28]. In chronic hepatitis B, ara-A, administered alone or in combination with interferon, inhibits viral replication [29–32] and in some patients brings about a disappearance of Dane particles from blood [29,30] and liver [30]. However, ara-A produces side effects, such as gastrointestinal and neurological disturbances, which can necessitate discontinuation of the therapy [33]; it also cause a marked lymphocytopenia which may affect the outcome of the treatment since succesful antiviral therapy appears to require the cooperation of the host immune response [34]. Selective concentration of ara-A or ara-AMP into hepatocytes, as achieved by coupling ara-AMP to L-SA, should reduce the side effects and improve the efficacy of the treatment.

The possibility exists that antibodies are produced against ara-AMP conjugates, even if they are prepared with homologous L-SA and in further experiments we will study this problem in laboratory animals. However, L-SA conjugates, even if complexed with antibodies, should maintain their original target and penetrate selectively into hepatocytes. Indeed the capacity of the hepatic receptor for galactosyl terminating glycoproteins to bind and internalize immune complexes has been shown [35]: the clearance of IgG–albumin complexes was mediated by the binding of galactosyl residues on IgG to the specific receptor on hepatocytes followed by uptake of the immune complexes by parenchymal liver cells.

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