Liver and biliary

Selective penetration and pharmacological activity of lactosaminated albumin conjugates of adenine arabinoside 5-monophosphate (ara-AMP) in mouse liver

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SUMMARY With the aim of improving the chemotherapeutic index of adenine arabinoside 5-monophosphate (ara-AMP) in the treatment of chronic hepatitis B, this drug was conjugated with lactosaminated serum albumin (L-SA), a neoglycoprotein which only enters into hepatocytes where it is digested in lysosomes. In mice, the L-[³H]SA-ara-AMP conjugates, intravenously injected, selectively penetrated the liver, only small quantities were taken up by cells of spleen, bone marrow, intestine, and brain. After administration of the conjugate to mice with Ectromelia virus hepatitis, ara-AMP was selectively concentrated in liver in a pharmacologically active form. If L-SA-ara-AMP conjugates behave in man as in mouse, their administration to patients with chronic hepatitis B should result in a selective concentration of ara-AMP in liver with a more efficient inhibition of virus replication accompanied by lower toxicity for other tissues.

In the therapy of some diseases – for example, tumours, infections caused by intracellular organisms – drug side effects might be reduced by coupling the drug to a macromolecular carrier which is selectively taken up by the cells where the pharmacological action is required. In these cells, conjugates will be pharmacologically effective if the bond linking the drug to the carrier does not suppress the activity of the drug or if the bond is broken down in lysosomes with consequent intracellular release of the drug.¹⁻³

In chronic hepatitis B infection, adenine-9-β-D arabinoside (ara-A) and its 5-monophosphate (ara-AMP) reduce the serum concentrations of virus DNA-polymerase⁴⁻⁹ with an improvement in liver function and histology¹⁰ and a loss of infectivity¹¹ in some patients. These drugs also produce neurological and haematological side effects,¹²⁻¹⁴ however, which might be avoided or reduced by selectively delivering them to hepatocytes. In order to obtain such targeting, ara-AMP was conjugated with either asialofetuin (AF)¹⁵ or with lactos-

aminated serum albumin (L-SA),^{16 17} two galactosyl-terminating glycoproteins which only enter, by a receptor mediated endocytosis, hepatocytes where they are digested in lysosomes.¹⁸ AF-ara-AMP and L-SA-ara-AMP conjugates, administered to mice with Ectromelia virus-hepatitis, inhibited virus DNA synthesis in liver without producing significant inhibition of cellular DNA synthesis in intestine and bone marrow.¹⁵⁻¹⁷ L-SA has a definite advantage over AF as a hepatotropic carrier of ara-AMP, because conjugates prepared with lactosaminated homologous albumin are devoid of humoral and cellular immunogenicity at least in mice.¹⁹

In the present experiments we (i) determined the organ distribution of radioactivity in mice injected with labelled L-SA-ara-AMP conjugates; (ii) studied the stability of the bond between ara-AMP and L-SA in mouse blood; and (iii) measured DNA synthesis in liver, intestine, and bone marrow of Ectromelia virus-infected mice at different times after administration of free ara-AMP and of a L-SA-ara-AMP conjugate; in previous studies^{16 17} similar experiments were performed but using only one time period after injection of these compounds.

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Methods

PREPARATION AND LABELLING OF L-SA

Lactose was coupled to ϵ -NH₂ of lysine residues of HSA (crystallised, essentially globulin free) (Sigma) and MSA (fraction V) (Sigma) by reductive amination with NaBH₃CN.^{20,21} The reaction was stopped when the molar ratio sugar/protein reached the value of 25 or 28 (L₂₅-MSA and L₂₈-HSA respectively). The albumin concentration was determined according to Lowry *et al.*²² The amount of sugar conjugated to protein was established by the phenol/sulphuric acid method of Dubois *et al.*²³ calibrated against galactose. L₂₈-HSA and L₂₅-MSA were labelled with [³H]formaldehyde (100 mCi/mmol) (NEN) in the presence of NaBH₃CN. This method assures a strong linkage of the introduced radioactive group.²⁴ Three hundred and fifty milligrams L₂₈-HSA and 280 μ Ci HCHO were dissolved in 12 ml of 0.2 M potassium phosphate buffer (pH 8), and incubated at 37°C for 15 minutes. NaBH₃CN (17 mg in 2 ml of the same buffer) was added and after a further 30 minutes incubation, at 37°C, the reaction was stopped by dialysis against water (specific activity: 1.3×10^6 dpm/mg). L₂₅-MSA was labelled by the same procedure (1.2×10^6 dpm/mg).

CONJUGATES OF L-SA WITH ARA-AMP

ara-AMP (Warner-Lambert) was conjugated with L₂₈-[³H]HSA, with L₂₅-[³H]MSA and with cold L₂₈-HSA using 1-ethyl-3-(dimethylaminopropyl) carbodiimide (Fluka) as previously described.¹⁵ Probably conjugation takes place mostly by formation of an amide bond between the ϵ -NH₂ group of lysine in L-SA and the phosphate group of ara-AMP as shown in an analogous reaction using carbodiimides for coupling thymidilic acid to albumin.²⁵ After gel chromatography on a Sephadex G-100 column, equilibrated and eluted with 0.05% NaCl solution, the conjugated material emerged in two peaks; the first peak contained polymers, whereas the second peak contained the monomeric form of the conjugate (the latter constituted about

70% of the whole preparation). The presence of polymers was because of the formation of covalent bonds between the protein molecules during carbodiimide coupling.²⁶ The fractions corresponding to the monomeric form of the conjugate were pooled, dialysed against water and lyophilised. The molar ratio ara-AMP/L-HSA was determined spectrophotometrically; it is indicated by the number under the drug – for example, L₂₈-HSA-ara-AMP₈ is a conjugate with a molar ratio ara-AMP/L₂₈-HSA of 8. We found that in contrast with previous data,^{16,17} ara-AMP coupling does not decrease the amounts of lactose bound to HSA or to MSA. In order to prepare a conjugate labelled in its ara-AMP moiety, 45 mg of ara-AMP containing 200 μ Ci ara-AMP (adenine, 2-³H) (22 Ci/mmol) (ICN, Irvine, California) were coupled to 22.5 mg L₂₈-HSA.¹⁵ The resulting conjugate L₂₈-HSA-ara-[³H]AMP₈ had a specific activity of 3×10^5 dpm/mg.

TISSUE DISTRIBUTION OF L-[³H]SA-ARA-AMP CONJUGATES

Female Swiss mice (28–30 g) were injected intravenously with conjugates dissolved in 0.9% NaCl (10 μ l/g body weight) at the doses indicated (Tables 1 and 2). At different times, the animals were bled from the retro-orbital plexus under ether anaesthesia, killed, and liver, a tract of intestine (8 cm long starting from pylorus), bone marrow from femurs, and in some experiments spleen and brain were rapidly removed and homogenised in water. For each dose and each time four animals were used. The total radioactivity of plasma and homogenates as well as the radioactivity of their acid soluble fraction after precipitation with 1 vol of 1.2 M perchloric acid was measured. The acid insoluble radioactivity was obtained by subtracting acid soluble from total radioactivity. The contribution of plasma trapped in the organs was calculated and subtracted as described by Regoeci²⁷ using monomeric HSA labelled with [¹⁴C] formaldehyde (55 mCi/mmol) (NEN) as a separate marker. [¹⁴C]HSA (1.4×10^6 dpm/mg) was injected

Table 1 Organ distribution of acid insoluble radioactivity 10 minutes after iv injection of L-[³H]SA-ara-AMP conjugates

Conjugate injected	Dose (μ g/body weight)	dpm (10^{-3})/mg DNA					dpm (10^{-3})/ml plasma
		Liver	Spleen	Bone marrow	Intestine	Brain	
L ₂₈ -[³ H]HSA-ara-AMP ₁₀	6	39.4 $\pm$ 1.3	0.2 $\pm$ 0.1	0.6 $\pm$ 0.2	1.2 $\pm$ 0.6	0.5 $\pm$ 0.1	38.2 $\pm$ 7.4
L ₂₈ -[³ H]HSA-ara-AMP ₁₀	35	51.7 $\pm$ 4.2	0.3 $\pm$ 0.1	3.4 $\pm$ 0.2	3.1 $\pm$ 0.5	1.4 $\pm$ 0.4	609.3 $\pm$ 20.6
L ₂₅ -[³ H]MSA-ara-AMP ₉	35	59.0 $\pm$ 1.4	—	1.3 $\pm$ 0.3	0.8 $\pm$ 0.2	—	604.6 $\pm$ 15.6

Each entry represents the mean value ($\pm$ SD) of results from four animals.

Table 2 Distribution of radioactivity in liver, intestine and bone marrow after injection of L_{28} - $[^3H]$ HSA-ara-AMP₁₀ (35 μ g corresponding to 4.5×10^4 dpm/g body weight)

Exp no	Route of administration of conjugate	Time after administration of conjugate (h)	Acid insoluble radioactivity dpm (10^{-3})/mg DNA			Acid soluble radioactivity dpm (10^{-3})/mg DNA			Radioactivity of plasma dpm (10^{-3})/ml		
			Liver	Intestine	Bone marrow	Liver	Intestine	Bone marrow	Acid insol	Acid sol	
1	iv	0.5	26.6 $\pm$ 0.4	0.9 $\pm$ 0.1	1.7 $\pm$ 0.1	28.9 $\pm$ 4.3	1.4 $\pm$ 0.4	0.4 $\pm$ 0	455.1 $\pm$ 29.5	4.4 $\pm$ 0.4	
2	iv	1	34.4 $\pm$ 3.1	1.8 $\pm$ 0	4.3 $\pm$ 0.4	58.9 $\pm$ 2.0	2.7 $\pm$ 0.6	0.6 $\pm$ 0.1	218.4 $\pm$ 23.7	10.0 $\pm$ 0.3	
3	iv	2	29.8 $\pm$ 2.8	1.2 $\pm$ 0.1	2.4 $\pm$ 0.5	85.6 $\pm$ 7.2	1.6 $\pm$ 0.1	0.6 $\pm$ 0.2	33.0 $\pm$ 7.7	11.2 $\pm$ 3.7	
4	iv	3	17.1 $\pm$ 0.5	0.8 $\pm$ 0	4.7 $\pm$ 2.0	83.2 $\pm$ 10.0	1.8 $\pm$ 0.2	0.8 $\pm$ 0.3	7.6 $\pm$ 0.2	13.5 $\pm$ 1.8	
5	iv	8	0.4 $\pm$ 0.4	0.1 $\pm$ 0.1	0.4 $\pm$ 0	103.7 $\pm$ 6.8	2.5 $\pm$ 0.3	0.4 $\pm$ 0.1	6.3 $\pm$ 0.6	3.1 $\pm$ 0.1	
6	ip	1	31.7 $\pm$ 0.5	1.7 $\pm$ 0.1	2.5 $\pm$ 0.6	60.0 $\pm$ 3.7	1.7 $\pm$ 0.1	0.7 $\pm$ 0.2	120.2 $\pm$ 67.0	6.0 $\pm$ 0.1	
7*	iv	1	25.2 $\pm$ 4.8	1.3 $\pm$ 0.2	6.3 $\pm$ 1.5	63.2 $\pm$ 4.1	2.2 $\pm$ 0.2	1.2 $\pm$ 0.3	280.8 $\pm$ 21.4	8.5 $\pm$ 0.5	

Each entry represents the mean value ($\pm$ SD) of results from four animals.

*Mice were infected with Ectromelia virus (2×10^5 pfu/g body weight) 46 hours before conjugate administration.

intravenously 10 minutes before animals were killed at the dose of 3 $\mu\text{g/g}$ body weight in 2.5 μl of saline. Acid soluble and insoluble radioactivities were expressed as dpm/mg DNA. Homogenate DNA was extracted by the method of Schneider and Greco²⁸ and measured according to Burton.²⁹

STABILITY OF THE BOND BETWEEN ARA-AMP AND L-SA IN MOUSE BLOOD

Female Swiss mice (28–30 g) were injected intravenously with $\text{L}_{28}\text{-}[^3\text{H}]\text{HSA-ara-AMP}_{10}$ or $\text{L}_{28}\text{-HSA-ara-}[^3\text{H}]\text{AMP}_8$ (35 $\mu\text{g}/10\text{ g}$ body weight). Four animals were used for each conjugate. One hour thereafter mice were killed, 40 μl of plasma were gel filtered on a 1.5 $\times$ 5 cm Sephadex G-25 medium column and the radioactivity of non-retarded fractions containing proteins was measured. The percentage of protein-bound dpm in 1 ml of plasma referred to total dpm injected was calculated. In $\text{L}_{28}\text{-}[^3\text{H}]\text{HSA-ara-AMP}_{10}$ the radioactive label (^3H formaldehyde) is linked to L-HSA by a bond which is stable and not enzymatically cleaved.²⁴ Therefore by comparing the percentage of protein-bound dpm in plasma of mice injected with $\text{L}_{28}\text{-}[^3\text{H}]\text{HSA-ara-AMP}_{10}$ with that of animals inoculated with $\text{L}_{28}\text{-HSA-ara-}[^3\text{H}]\text{AMP}_8$ stability of the bond linking ara-AMP to L-HSA could be evaluated.

In these experiments protein-bound radioactivity of conjugates could not be measured by precipitation with trichloroacetic, sulphosalicylic or perchloric acid because these precipitants cause a partial cleavage of the bond between ara-AMP and L-HSA.

EFFECT OF FREE OR CONJUGATED ARA-AMP ON [METHYL- ^3H] THYMIDINE INCORPORATION INTO DNA IN LIVER, INTESTINE, AND BONE MARROW OF ECTROMELIA VIRUS-INFECTED MICE

To study the action of free and conjugated ara-AMP on DNA synthesis in hepatocytes we used Ectromelia virus-infected mice (as in previous experiments^{15–17}), because in normal mice thymidine incorporation into liver DNA takes place mainly in sinusoidal cells³⁰ and it is scarcely affected by free ara-A or ara-AMP even in the presence of inhibitors of ara-A deamination. Forty six hours after infection with Ectromelia virus (2×10^5 pfu/g body weight) thymidine incorporation into liver DNA is four to six times higher than in non-infected mice³¹ and is sensitive to ara-A and ara-AMP in the presence of inhibitors of ara-A deamination (see below). At this point of infection the largest amount of thymidine incorporation into liver DNA is presumably because of virus DNA synthesis most of which occurs in hepatocytes.^{16, 31}

For each experiment 21 Swiss female mice (28–30

g) were injected intravenously with purified^{32, 33} Ectromelia virus (Hampstead mouse strain) (2×10^5 pfu/g body weight) and divided in three groups of seven animals each. All the compounds were dissolved in NaCl 0.9% and injected in a volume of 10 $\mu\text{l/g}$ body weight. In the experiment in which the interval between the injection of ara-AMP (free or conjugated) and that of [methyl- ^3H] thymidine was one hour (see Results), 9-erythro-(2-hydroxyl-3-nonyl)-adenine (EHNA) was administered ip to all three groups of mice (3 $\mu\text{g/g}$ body weight) 44–15 hours after infection. EHNA is a short-lasting inhibitor of ara-A deamination. In fact an ip injection, in mice, of this compound (3 $\mu\text{g/g}$ body weight) initially inhibits ara-A deamination by 95% with recovery to 50% of control values within 30 minutes.³⁴ Administration of EHNA is necessary because ara-A (and ara-AMP) is several times less active in rodents than in primates³⁵ and consequently these drugs injected alone, even at high doses, do not affect DNA synthesis in liver and intestine of Ectromelia virus-infected mice.¹⁵ EHNA administered alone to Ectromelia virus-infected mice, is ineffective on DNA synthesis in liver, intestine, and bone marrow.¹⁶ Fifteen minutes after EHNA inoculation, mice of group 1 (controls) were injected intravenously with saline, mice of group 2 with free ara-AMP (7.5 $\mu\text{g/g}$ body weight) and mice of group 3 with $\text{L}_{28}\text{-HSA-ara-AMP}_8$ (35 $\mu\text{g/g}$ body weight). After one hour all mice were ip inoculated with [methyl- ^3H] thymidine (spec act 24 Ci/mmol; Amersham) (0.7 $\mu\text{Ci/g}$ body weight). Thirty minutes thereafter (46 hours after infection) animals 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 by the method of Schneider and Greco,²⁸ the radioactivity was determined and the concentration measured according to Burton.²⁹ The experiments in which the intervals, between the administration of free or conjugated ara-AMP and that of [methyl- ^3H] thymidine, lasted two to three hours were performed using the same procedure except that EHNA was injected 43.15 or 42.15 hours after infection respectively.

Results

L-SA-ara-AMP conjugates do not display any recognisable sign of toxicity even at a dose of 110 $\mu\text{g/g}$ body weight.¹⁹ This is the highest dose tested, and three times larger than the highest dose used in the experiments reported here.

Table 1 shows the organ distribution of acid insoluble radioactivity 10 minutes after administration of $\text{L-}[^3\text{H}]\text{SA-ara-AMP}$ conjugates. At this

time acid soluble radioactivity is negligible (less than 5% of total) because hydrolysis of conjugates has just started. The amount of acid insoluble radioactivity, in reference to DNA, is several times higher in liver than in spleen, bone marrow, intestine, and brain. No substantial difference was found in liver radioactivity after administration of conjugates either prepared with heterologous (human) or homologous L-SA.

Table 2 shows acid insoluble and soluble radioactivity in liver, intestine, and bone marrow at times ranging from 30 minutes to eight hours after L_{28} - $[^3H]$ HSA-ara-AMP₁₀ administration (35 μ g/g body weight). Up to three hours both acid insoluble and soluble radioactivities are many times higher in liver than in intestine and in bone marrow. Acid soluble radioactivity is several fold higher in liver than in other organs even at eight hours. This may be because of radioactive peptides, resulting from partial hydrolysis of the conjugate, which hardly cross the external membrane of hepatocytes. Different modes of administration of the conjugate (iv or ip route) do not affect the distribution of acid insoluble and soluble radioactivities in the organs, at least at one hour.

One hour after mice were injected intravenously with L_{28} -HSA-ara- $[^3H]$ AMP₈ (at the dose of 35 μ g/g body weight), the protein-bound radioactivity in 1 ml of plasma was $14.3\% \pm 0.5$ (SD) of the total radioactivity administered. This value was very close to that ($16.8\% \pm 1.7$) found after administration of L_{28} - $[^3H]$ HSA-ara-AMP₁₀ at the same dose. As in the latter conjugate $[^3H]$ formaldehyde is attached to L-SA by a very strong bond which is not enzymatically broken down,²⁴ this indicates that ara-AMP is linked to L-HSA by a bond which is stable or undergoes only a slight hydrolysis in mouse blood.

The Figure shows the effect of free and conjugated ara-AMP on DNA synthesis, in liver, intestine, and bone marrow of Ectromelia virus-infected mice, at different times after their administration. Free ara-AMP was injected at a dose of 7.5 μ g/g body weight which corresponds to that administered to patients with chronic hepatitis B (5–15 mg/kg/day).⁸ The drug produced significant inhibitions in the organs at one hour. The inhibition in the liver was greater than in bone marrow but lower than in intestine. At two hours there was a small and not significant inhibition in bone marrow and liver. At three hours there was no longer inhibition in any of the three organs. After injection of conjugated ara-AMP, in a dose about five times lower than that of the free drug, significant inhibition was found in liver at one and two hours but not at three hours. In bone marrow a small

Figure Effect of free ara-AMP (I) and of L_{28} -HSA-ara-AMP₈ (II) on [methyl- 3H] thymidine incorporation into DNA in liver (□), intestine (Δ) and bone marrow (●) of Ectromelia virus infected mice. ara-AMP and L_{28} -HSA-ara-AMP₈ were injected iv at dose of 7.5 and 35 μ g/g b w respectively. 35 μ g conjugate contains 1.4 μ g ara-AMP. Results (dpm/mg DNA) were statistically evaluated by means of Student's t test. + means that the difference between [methyl- 3H] thymidine incorporation into DNA in treated and control mice was significant ($p < 0.05$).

inhibition was found at one hour but not at two and three hours. There was practically no inhibition in intestine at any time. Lactosaminated albumin did not exert any action on DNA synthesis in the three organs and did not affect the action of free ara-AMP. This drug injected intravenously (7.5 μ g/g body weight) simultaneously with L_{28} -HSA (35 μ g/g body weight), produced after one hour an inhibition of DNA synthesis in liver, intestine, and bone marrow of 49%, 55%, and 50% respectively.

Discussion

These experiments show that in mice injected with L- $[^3H]$ SA-ara-AMP conjugates the radioactivity in liver is higher than in other organs. In L- $[^3H]$ SA-ara-AMP, the radioactive label is attached to the protein part of the conjugate. As the bond linking ara-AMP to L-SA is stable in mouse blood,

however, (see Results), the greater acid insoluble radioactivity in liver very probably corresponds to a higher hepatic uptake of the whole conjugate and not only of its protein moiety.

Experiments on the action of free and coupled ara-AMP on DNA synthesis, confirm our previous results^{16 17} and show that, after administration of L-SA-ara-AMP conjugates to mice with Ectromelia virus hepatitis, ara-AMP is selectively concentrated in liver in a pharmacologically active form. Because the ester bond linking phosphate to ara-A may be broken down in lysosomes, it cannot be excluded that the drug released from the conjugate was ara-A and not ara-AMP.

In conclusion, L-SA-ara-AMP conjugates specifically penetrate into mouse liver where ara-AMP (or ara-A) is released with consequent hepatic targeting of this drug.

If these conjugates behave in man as in mouse, their administration to patients with chronic hepatitis B should result in a selective concentration of ara-AMP in liver with a more efficient inhibition of virus replication accompanied by a lower toxicity for the other tissues. Reduction of side effects should permit prolongation of the cycles of antiviral treatment with an improvement of the therapeutic results.

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