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A study on the pharmacokinetics in mouse of adenine-9-β-D-arabinofuranoside 5-monophosphate conjugated with lactosaminated albumin[1,2]

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Summary. In plasma of mice injected with adenine-9-β-D-arabinofuranoside monophosphate (ara-AMP) coupled to human lactosaminated serum albumin (L-HSA) some of the ara-AMP molecules are enzymatically released, whereas others remain linked to L-HSA. Evidence has been obtained that ara-AMP is not deaminated when it is conjugated to L-HSA, in contrast to the free drug which is rapidly metabolized to its hypoxanthine derivative.
Key words. Adenine arabinoside 5-monophosphate; drug targeting; hepatitis B.

In order to increase the chemotherapeutic index of adenine-9-β-D-arabinofuranoside monophosphate (ara-AMP) in the treatment of chronic hepatitis B, this drug was conjugated with human lactosaminated albumin (L-HSA)[3-6], a neoglycoprotein with terminal galactosyl groups which is taken up only into hepatocytes, where it is digested in lysosomes[7-9]. In mouse, L-HSA-ara-AMP conjugates enter liver cells almost exclusively; only very small quantities are taken up by the other organs[6]. Administered to mice with *Ectromelia* virus hepatitis they inhibit virus DNA synthesis in liver without significantly inhibiting cellular DNA synthesis in intestine and bone marrow[3-6]. L-SA-ara-AMP conjugates prepared with homologous L-SA are devoid of humoral and cellular immunogenicity at least in mice[10]. For details of abbreviations see under references[2].

In order to obtain some data on the pharmacokinetics of L-HSA-ara-AMP conjugates, in the present experiments we 1) developed a radioimmunoassay (RIA) enabling us to measure ara-AMP linked to L-HSA in mouse plasma; 2) studied the stability in vitro of the bond between ara-AMP and L-HSA in mouse and human plasma; 3) investigated the rate of disappearance of protein-bound radioactivity from plasma of mice injected with conjugates labeled in the protein or in the drug moiety.

Materials and methods. Lactose was coupled to HSA (crystallized, essentially globulin free) (Sigma) by reductive amination with $NaBH_3CN$[11,12]. The reaction was stopped when the molar ratio sugar/protein reached the value of 30 (L_{30}-HSA). Ara-AMP (Warner-Lambert) was conjugated with L_{30}-HSA by a slight modification of the method of Fiume et al.[13]. In different conjugate preparations the molar ratio ara-AMP/L-HSA, determined spectrophotometrically, ranged from 6.1 to 6.4; it is indicated by the number under the drug (e.g. L_{30}-HSA-ara-$AMP_{6.1}$). With the same procedure, hypoxanthine-9-β-D-arabinofuranoside 5-monophosphate (ara-HxMP) (ICN) was coupled to L_{30}-HSA. The resulting conjugate had a molar ratio ara-HxMP/protein of 4.7.

Three radioacitve conjugates, one labeled in the protein and two in the drug moiety, were prepared. The first one (L_{30}-[^{3}H]HSA-ara-$AMP_{6.2}$) was obtained by coupling ara-AMP to L_{30}-HSA which has been previously labeled with [^{3}H]formaldehyde (100 mCi/mole) (NEN) in the presence of $NaBH_3CN$[14] according to the procedure previously described[6]. The specific activity of the conjugate was 1.3×10^6 dpm/mg. To prepare the conjugates radioactive in the drug moiety, tritiated ara-AMP (either [2-^{3}H adenine] 22 Ci/mmole, ICN or [2,8-^{3}H adenine] 16 Ci/mmole, Amersham) was diluted with the cold drug and coupled with L_{30}-HSA. The resulting conjugates L_{30}-HSA-ara-[2-^{3}H]$AMP_{6.1}$ and L_{30}-HSA-ara-[2,8-^{3}H]$AMP_{6.4}$ had a sp.act. of 5.8×10^5 and 6.8×10^5 dpm/mg respectively.

In order to study the cleavage of the bond between ara-AMP and L-HSA in mouse or human plasma, in vitro, 180 or 750 μg L_{30}-HSA-ara[2,8-^{3}H]AMP in 50 μl of saline (NaCl 0.9%) were added to 950 μl of heparinized plasma or saline pre-heated to 37°C. After different times (at 37°C, with shaking) 40 μl of the mixture was gel filtered on a 1.5 × 5 cm Sephadex G-25 Medium

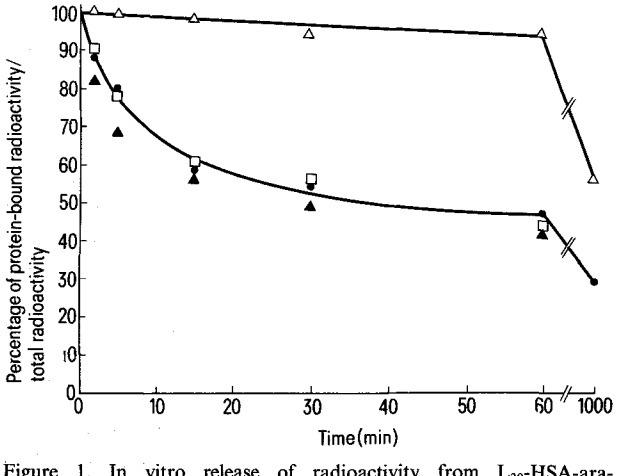

Figure 1. In vitro release of radioactivity from L_{30}-HSA-ara-[2,8^{3}H]$AMP_{6.4}$. △, in saline (750 μg conjugate/ml); ●, in mouse plasma (750 μg/ml); ▲, in mouse plasma (180 μg/ml); □, in human plasma (750 μg/ml).

column, equilibrated and eluted with phosphate buffered saline (PBS) 0.15 M, pH 7.4. The radioactivity of the non retarded fraction (containing protein-bound radioactivity) was measured. Protein-bound radioactivity was not measured by precipitation of the conjugate with trichloroacetic, sulphosalicylic or perchloric acid since these precipitants cause a partial cleavage of the bond between ara-AMP and L-HSA.

For the studies on plasma levels of conjugates in vivo, female Swiss mice weighing 28–30 g were used. Conjugates were injected i.v. at 35 µg/g b.wt, a dose which had been used in previous experiments[6]. At different times, blood was taken from the retroorbital plexus under ether anesthesia, heparinized and rapidly centrifuged in the cold; samples of 40–200 µl of plasma were immediately gel filtered on the 1.5 × 5 cm Sephadex G-25 Medium column. Radioactivity determination as well as RIA were performed on samples of the non retarded fraction. RIA was carried out (without any delay after gel filtration) by using ara-[2,8-^{3}H]adenine (7.6 Ci/mmole, Amersham) and a serum binding this nucleoside. In order to obtain such a serum 12 male Wistar rats weighing about 300 g were immunized[15] with three different conjugates (four for each conjugate). A conjugate was prepared by coupling ara-A glutarate[13] to desialylated[16] fetuin (AF) via its hydroxisuccinimide ester[13] (AF-ara-A-glutarate). The molar ratio drug/protein of this conjugate was 8. The other two conjugates (L_{30}-HSA-ara-AMP_7 and AF-ara-AMP_4) were prepared by carbodiimide coupling of ara-AMP[13].

The ara-A binding capacities of the sera were measured according to Farr[17]. Rats immunized with the conjugates prepared by coupling ara-AMP to AF or L-HSA practically did not produce antibodies binding either ara-A or ara-AMP. The ara-A binding capacities of sera from rats immunized with AF-ara-A glutarate ranged from 177 to 936 pmoles araA/ml. The serum with highest titre was selected for the immunoassay. Free ara-AMP binds, but with a low affinity, to anti-ara-A antibodies; 5 or 20 pmoles of ara-AMP displace only 0.2 and 0.5 pmoles respectively of ara-[2,8-^{3}H]A in the assay.

To obtain a standard curve, different amounts of L_{30}-HSA-ara-$AMP_{6.1}$ (0.44–3.5 µg) were dissolved in 2.5 ml PBS 0.15 M, pH 7.4 and gel filtered with the same buffer on 1.5 × 5 cm Sephadex G-25 Medium column, in order to remove small amounts of free drug which might still be present in the conjugate preparation. 400 µl of the non retarded fraction (= 3.5 ml) which contained 50–400 ng of conjugate (corresponding to 1.5–12 ng of coupled ara-AMP) was mixed with 1 µl of ara-A binding serum and 1 pmole of ara-[2,8^{3}H]A, brought to 500 µl with PBS 0.15 M, pH 7.4, incubated at 2–4 °C for 3 h and processed as described[15]. The amounts of coupled ara-AMP (ng/tube) were plotted with the ratios of dpm precipitated in the absence of conjugate to those precipitated in its presence. RIA is expected to detect only a part of ara-AMP molecules coupled to L-HSA since very probably the antibodies cannot bind to some conjugated molecules of drug because of a steric hindrance.

Results and discussion. RIA evaluation. The curve of RIA was linear over the tested range (1.5–12 ng) of conjugated ara-AMP with a slope of 50°. 400 µl of the nonretarded fractions of gel filtered samples of plasma obtained from five untreated mice, and separately tested, did not interfere with the immunoassay. In 10 plasma samples containing known amounts of conjugated ara-AMP, the values obtained after gel filtration and RIA of non retarded fractions were 92–110% of the expected. Since ara-AMP is rapidly converted to the hypoxanthine derivative in the plasma of man and of experimental animals[18–20], we also tested whether ara-HxMP coupled to L-HSA interferes with the assay. Conjugated ara-HxMP, up to 100 ng/tube (the maximum tested), did not produce any interference. Moreover 400 µl of the non retarded fractions of gel filtered plasmas taken from two mice, 2 min after the injection of 2.1 µg/g b.wt of conjugated ara-HxMP (a dose twice as large as that of conjugated ara-AMP used in these experiments) did not interfere with the binding of antibodies to ara-[^{3}H]A.

Experiments in vitro. Figure 1 shows a partial release of radioactivity from the L_{30}-HSA-ara-[2,8-^{3}H]$AMP_{6.4}$ conjugate in the presence of mouse or human plasma. The cleavage is mainly enzymatic since under the same conditions, but in the absence of

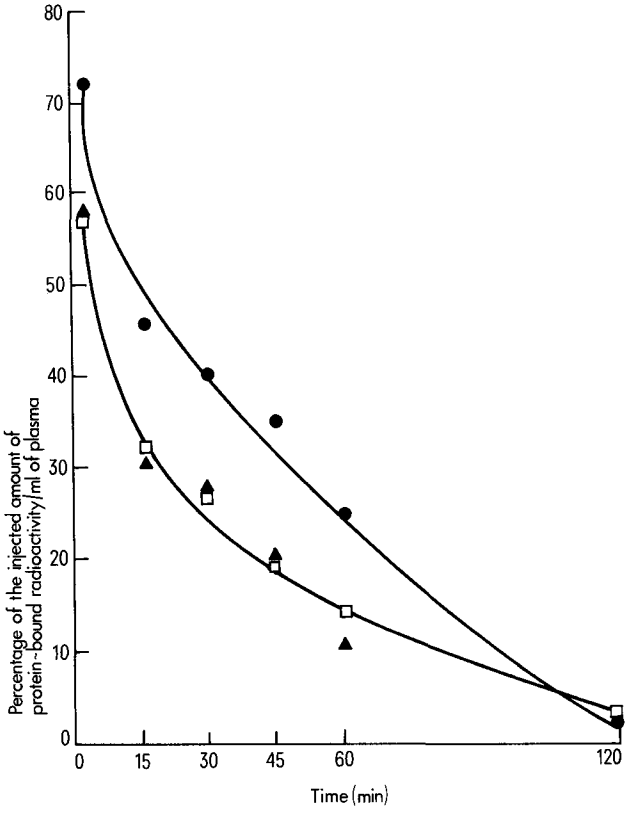

Figure 2. Protein-bound radioactivity in plasma of mice injected i.v. with conjugates labeled in the protein or in the drug moiety (35 µg/g b.wt). ●, L_{30}-[^{3}H]HSA-ara-$AMP_{6.2}$; □, L_{30}-HSA-ara-[2^{3}H]$AMP_{6.1}$; ▲, L_{30}-HSA-ara-[2,8^{3}H]$AMP_{6.4}$.

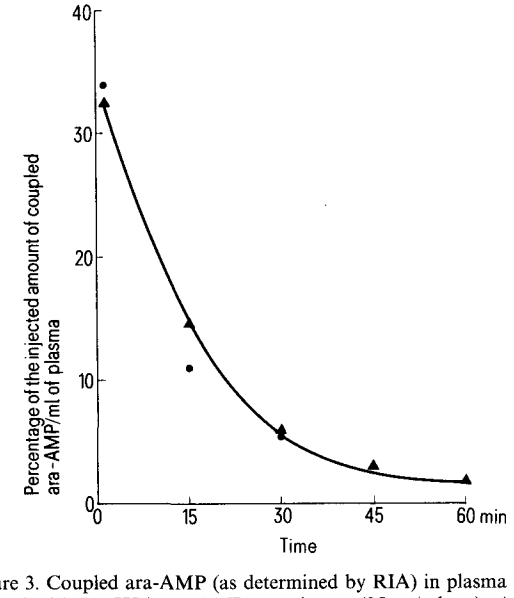

Figure 3. Coupled ara-AMP (as determined by RIA) in plasma of mice injected with L_{30}-HSA-ara-$AMP_{6.1}$ conjugate (35 µg/g b.wt). ▲, in the absence of deoxycoformycin; ●, in the presence of deoxycoformycin administered i.p. at a dose of 2 µg/g b.wt, 15 min before the conjugate.

plasma, the release of radioactivity is very small. The release of radioactivity is rapid at the beginning but after 5–10 min, notwithstanding the concentrations of conjugate, it slows down and practically stops when the protein-bound radioactivity is about 50% of the initial value. If after 60 min of incubation of L_{30}-HSA-ara-[2,8-^{3}H]$AMP_{6.4}$ in mouse plasma (750 µg/ml), new labeled conjugate is added, the release of radioactivity from the conjugate starts again, following the same curve as in the first 60 min (experiment not shown in fig. 1).

These in vitro experiments indicate that some (40–50%) of the conjugated ara-AMP molecules remain linked to L-HSA in mouse plasma. The inhibition of DNA synthesis, produced by L-SA-ara-AMP conjugates selectively in the liver of mice with *Ectromelia* virus hepatitis, is very probably caused by these drug molecules which are not released freely in plasma and can be transported by L-SA selectively into the hepatocytes.

It is probable that the ara-AMP molecules which remain linked to L-HSA are protected by a steric hindrance from the enzymes which cleave the linkage between the drug and the carrier protein. However, it is not possible to exclude the possibility that different drug-protein bonds are formed during the conjugation and that the ara-AMP molecules which remain linked to L-HSA are coupled by a bond which is not enzymatically cleaved.

Experiments in vivo. In contrast to previous results[6] the values of protein-bound radioactivity in mouse plasma after injection of the conjugates labeled in the ara-AMP moiety are lower than those after administration of an equal dose of L_{30}-[^{3}H]HSA-ara-$AMP_{6.2}$ (fig. 2). In the latter the radioactive label is linked to protein by a bond which is very strong and not enzymatically cleaved[14]. A splitting of the bond between the drug and the protein (or between ara-A and the phosphate) accounts for this finding as has been demonstrated by the experiments in vitro (fig. 1).

Figure 3 shows the curve of disappearance from plasma of conjugated ara-AMP as identified by RIA. Deoxycoformycin, a drug which when administered i.p. to mice at the dose of 2 µg/g completely inhibits ara-A deamination for more than 5 h[21], does not increase the plasma levels of coupled ara-AMP (fig. 3). This suggests that ara-AMP conjugated to L-HSA is not deaminated in mouse plasma in contrast to free ara-A and ara-AMP which are very rapidly metabolized to the hypoxanthine derivative[18–20]. Coupled ara-AMP, detected by RIA, is present in plasma in percentages of the administered dose (fig. 3) which are lower than the percentages of protein-bound radioactivity after injection of the conjugates labeled in the drug moiety (fig. 2). This may occur because those ara-AMP molecules which are protected by steric hindrance from the enzymes which cleave the bond with L-HSA (as was suggested above) may be the same which are not accessible to antibodies and consequently are not detected by RIA (see 'materials and methods').

In conclusion, the present experiments show that in plasma of mice some ara-AMP molecules are enzymatically released from L-HSA conjugates whereas some of them remain linked to the carrier, probably because they are protected by steric hindrance from the activity of enzymes. Evidence has been obtained that ara-AMP is not deaminated when it is conjugated to L-HSA.

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2 The abbreviations used are: ara-AMP, adenine-9-β-D-arabinofuranoside 5-monophosphate; ara-HxMP, hypoxanthine-9-β-D-arabinofuranoside 5-monophosphate; AF, asialofetuin; L-HSA, human lactosaminated serum albumin; L-HSA-ara-AMP, conjugates of L-HSA with ara-AMP; L-[^{3}H]HSA-ara-AMP, conjugates tritiated in the protein moiety; L-HSA-ara-[2,8^{3}H]AMP and L-HSA-ara-[2^{3}H]AMP, conjugates tritiated in the adenine moiety of ara-AMP. The molar ratios lactose/HSA and ara-AMP/HSA are indicated by subscripts; for example L_{30}-HSA-ara-$AMP_{6.2}$ is a conjugate with a molar ratio of lactose/HSA of 30 and of ara-AMP/HSA of 6.2.

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Calcium accelerates cholesterol phase transitions in analog bile

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Summary. Analog bile supersaturated with cholesterol was constituted, filtered and divided into equal portions containing no calcium or calcium, 2.5–15 mM. Aliquots were removed over the next 48 h and filtrates analyzed for cholesterol, bile acid and lecithin. Calcium accelerated cholesterol loss from solution in a dose-related fashion.

Key words. Calcium; cholesterol supersaturation; lithogenicity.

Cholesterol supersaturation of bile is always present in patients with cholesterol gallstones[1]. However, since cholesterol supersaturation also occurs in healthy man, lithogenicity is probably best defined as an inability to maintain cholesterol in solution in a supersaturated state[2–3]. In spite of supersaturation in both groups, cholesterol microcrystals are consistently present in bile