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DRUG TARGETING IN ANTIVIRAL CHEMOTHERAPY

A CHEMICALLY STABLE CONJUGATE OF 9-β-D-ARABINOFURANOSYL-ADENINE 5'-MONOPHOSPHATE WITH LACTOSAMINATED ALBUMIN ACCOMPLISHES A SELECTIVE DELIVERY OF THE DRUG TO LIVER CELLS*

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Abstract—With the aim of improving the chemotherapeutic index of 9-β-D-arabinofuranosyl-adenine 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. We used a L-SA-ara-AMP conjugate which, in contrast to those previously employed, has the advantage of remaining soluble after lyophilization. We found in mice that: (I) this new conjugate was quite stable in the bloodstream where only a small part of ara-AMP was released; (II) after administration of the conjugate labelled in the drug moiety both acid insoluble and soluble radioactivities were several times higher in liver than in other organs; (III) in mice with Ectromelia virus hepatitis, the conjugate inhibited virus DNA synthesis in liver without affecting cellular DNA synthesis in intestine and bone marrow; (IV) the conjugate did not display any recognizable sign of acute toxicity even at doses several fold higher than those pharmacologically active; and (V) when prepared with homologous albumin it was not immunogenic.

According to the lysosomotropic approach to antiviral chemotherapy [1], ara-AMP† was either conjugated with AF [2] or with L-SA [3, 4], in order to introduce it selectively in hepatocytes and to reduce its side effects [5–10] in the treatment of chronic hepatitis B [11–18]. AF and L-SA are galactosyl terminating glycoproteins which after interaction with Ashwell's receptor specifically penetrate into parenchymal liver cells where they are delivered to lysosomes [19–21]. Conjugation was performed by allowing ara-AMP activated by a water soluble carbodiimide to react with AF or L-SA in an acidic medium [2]. In mice with Ectromelia virus hepatitis, AF-ara-AMP and L-SA-ara-AMP inhibited virus DNA synthesis in liver without producing significant inhibition in intestine and bone marrow [2–4]. L-SA had a definite advantage over AF as a hepatotropic carrier of ara-AMP, because conjugates prepared with lactosaminated homologous albumin were devoid of humoral and cellular immunogenicity at least in mice [22]. A drawback of the L-SA-ara-AMP conjugate was a rapid loss of its solubility which occurred because of polymerization after lyophilization [23]. We found that the polymerization was caused by the phosphoanhydride bond which links a large part of the coupled ara-AMP molecules to L-SA [23]. This bond is very reactive and in the presence of an $-NH_2$ group undergoes aminolysis with the formation of a carboamide bond and release of the phosphate [24]. In the lyophilized conjugate a phosphoanhydride bond can react with a lysine ε-NH_2 group of another L-SA-ara-AMP molecule thus causing protein aggregation.

By increasing the pH of the coupling reaction from 5 to 7.5, a new L-SA-ara-AMP conjugate has recently been obtained [23] which does not polymerize and remains soluble after lyophilization. The bonds formed in this conjugate are not known but they are at least in part different from that (those) of the former conjugate [23]. Since the type of bond between a drug and a carrier may strongly affect the biological activity of the conjugate [25, 26], the present experiments were undertaken to ascertain whether liver targeting of ara-AMP can also be obtained with the L-SA-ara-AMP prepared at alkaline pH. Using mice we studied: (I) the stability of the bond(s) between the drug and L-SA in blood; (II) the distribution in liver, spleen, intestine and kidney of both acid insoluble and soluble radioactivities after administration of the conjugate labelled in the ara-AMP moiety; (III) the effect of the conjugate on DNA synthesis in liver, intestine and bone marrow of mice with Ectromelia virus hepatitis; (IV) the acute toxicity of conjugate; (V) its immunogenicity when prepared either with homologous or heterologous L-SA.

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† Abbreviations: ara-AMP, 9-β-D-arabinofuranosyl-adenine 5'-monophosphate; AF, asialofetuin; L-SA, lactosaminated serum albumin; L-HSA, human L-SA; L-MSA, mouse L-SA; L-SA-ara-AMP, conjugates of L-SA with ara-AMP; L-[^{3}H]SA-ara-AMP, conjugates tritiated in the albumin moiety; L-SA-ara-[2,8-^{3}H]AMP, conjugates tritiated in the adenine moiety of ara-AMP. The molar ratio lactose/SA and ara-AMP/SA are indicated by subscripts: for example L_{30}-HSA-ara-AMP_{14} is a conjugate with a molar ratio of lactose/HSA of 30 and of ara-AMP/HSA of 14. ECDI, 1-ethyl-3-(dimethyl-aminopropyl)carbodiimide; PBS, 0.15 M phosphate buffered saline; S.E., standard error.

MATERIALS AND METHODS

Conjugates. α-Lactose (Sigma) was coupled to ε-NH_2 lysine residues of HSA (crystallized, essentially globulin free) (Sigma) and MSA (fraction V) (Sigma) by reductive amination with $NaBH_3CN$ [21, 27]. The reaction was stopped when the molar ratio sugar/protein reached the value of 30. Ara-AMP was coupled to L_{30}-HSA by using 1-ethyl-3-(dimethylaminopropyl) carbodiimide (ECDI) (Fluka) at pH 7.5 [23]. The whole complex product including the monomer of the conjugate and the polymers, which were formed as a side reaction of carbodiimide coupling [28], was collected. To remove uncoupled ara-AMP, ECDI and its urea derivative the conjugate was dialyzed against 0.9% NaCl. Subsequently, it was concentrated to 1 g/10 ml in minicon B15 cells (Amicon) and fractions of 1 ml (containing 100 mg conjugate and 9 mg NaCl) were lyophilized. NaCl could not be removed because the conjugate was partially insoluble in water. When required each fraction was dissolved in 1 ml water and further diluted if necessary with NaCl 0.9%. The molar ratio ara-AMP/L_{30}-SA in the conjugate was determined by calculating the ara-AMP concentration from the extinction at 260 nm ($E^{1\%} = 420$) after subtracting the absorption of albumin at this wavelength ($E^{1\%} = 3.86$). Albumin concentration was determined according to Lowry *et al.* [29]. In order to measure the molar ratio ara-AMP/L_{30}-HSA in the lyophilized conjugate, 5–10 mg conjugate in 1 ml 0.9% NaCl was gel filtered on a 1.35 × 65 cm Sephadex G-100 column, equilibrated and eluted with the same saline, so that any free ara-AMP (that might be present) could be removed. Sephadex G-100 was used since the conjugate strongly adsorbs to other gels.

Radioactive L-SA-ara-AMP conjugates, labelled in the protein or in the drug moiety, were also prepared. The first type (L_{30}-[^{3}H]SA-ara-AMP) was obtained by coupling ara-AMP to L_{30}-HSA or to L_{30}-MSA previously labelled with [^{3}H]formaldehyde (100 mCi/mmol) (NEN) in the presence of $NaBH_3CN$ [30]. The specific activity of radioactive L_{30}-[^{3}H]SA ranged from 1.7×10^6 dpm/mg to 1.3×10^7 dpm/mg according to the different concentrations of reactants employed. To prepare the conjugate radioactive in the drug moiety, tritiated ara-AMP [2,8-^{3}H-adenine] (16 Ci/mmol, Amersham) was diluted to 10 μCi/mg with the cold drug and coupled to L_{30}-HSA. The resulting conjugate L_{30}-HSA-ara-[2,8-^{3}H]AMP_{14} had a specific activity of 8×10^5 dpm/mg which corresponded to 1.4×10^4 dpm/μg of coupled drug.

Acute toxicity of L_{30}-HSA-ara-AMP_{14} conjugate. Seventy-five female Swiss mice weighing 28–30 g were divided into three groups of 25 animals each. Mice of group 1 were not treated; those of group 2 were slowly injected i.v. with 400 μl saline (0.9% NaCl); those of group 3 received a slowly administered i.v. injection of 40 mg L_{30}-HSA-ara-AMP_{14} in 400 μl saline. Injected solutions had been sterilized by filtration through a 0.45 μM Millipore filter. After injection animals were observed three times daily for a 10-day period during which their weight and the food intake were measured each day.

Immunogenicity of conjugates. Female Swiss mice (28–30 g) were injected s.c. with 200 μl of complete Freund's adjuvant emulsion containing 100 μg of L_{30}-MSA-ara-AMP_{14} or L_{30}-HSA-ara-AMP_{14} administered in 2 different sites (100 μl in each site). Fifteen animals were used for each conjugate. After 1 month mice received a s.c. booster dose of 200 μg of the conjugate in 200 μl of 0.9% NaCl. A week later the animals were bled from the retroorbital plexus under ether anaesthesia. To 4 male Wistar rats (400–450 g) a total volume of 500 μl of complete Freund's adjuvant emulsion containing 500 μg of L_{30}-MSA-ara-AMP_{14} was injected s.c. at 2 different sites. After 1 month the animals received a s.c. booster dose of 1 mg of conjugate in 1 ml of 0.9% NaCl; a week later they were bled. Antibodies binding the conjugates were measured by the ammonium sulphate method according to Minden and Farr [31]. The monomeric forms of L_{30}-[^{3}H]MSA-ara-AMP_{14} (spec. act. 1.2×10^7 dpm/mg) and of L_{30}-[^{3}H]HSA-ara-AMP_{14} (spec. act. 1.1×10^7 dpm/mg) were used as radioactive antigens. Polymers of radioactive antigens were removed because they are in part insoluble in 50% ammonium sulphate. Radioactive conjugate (50 mg) dissolved in 3 ml 0.9% NaCl was gel chromatographed on a Sephadex G-100 column (1.9 × 130 cm) equilibrated and eluted with the same saline. The conjugate emerged in two peaks. The fractions of the second part of the second peak, which contained only the monomeric form of conjugate, were pooled and used as antigen. Sera from treated animals were tested in amounts up to 50 μl. When smaller amounts of tested sera were used, serum volume was brought to 50 μl with normal non immune mouse serum as carrier. 450 μl of 0.15 M phosphate-buffered saline (PBS) pH 7.4, and 0.5 μg radioactive antigen were added to the serum. The mixture was incubated at 2–4° for 3 hr and then precipitated with 500 μl of saturated ammonium sulphate solution (pH 7.4). The precipitate sedimented by centrifugation at 6000 *g* and at 2–4° was dissolved in 3 ml PBS and precipitated again 3 times with an equal volume of the ammonium sulphate solution. The final precipitate was dissolved in 1 ml of Lumasolve (Lumac) and the radioactivity was determined after addition of 10 ml Dimilume-30 (Packard). In the absence of immune serum 97 + 10 (S.E.) dpm were precipitated. The sensitivity limit of the assay was about 0.5 μg IgG per ml of serum.

RESULTS

Chemical characteristics of the conjugate

The molar ratio ara-AMP/L_{30}-HSA in 12 conjugate preparations ranged from 13 to 15. Solubility and molar ratio of the conjugate, kept at 0–4°, were not decreased up to 6 months after preparation (maximum interval tested). The bonds linking ara-AMP to L_{30}-HSA were acid stable: they were resistant to protein precipitants such as 5% trichloroacetic acid, in contrast to those of conjugate prepared at acidic pH [4]. As indicated by SDS gel electrophoresis, the conjugate was composed of the monomer as well as of polymers of L_{30}-HSA which were formed as a side reaction of carbodiimide coupling [28]. The percentage of monomer, dimer, trimer,

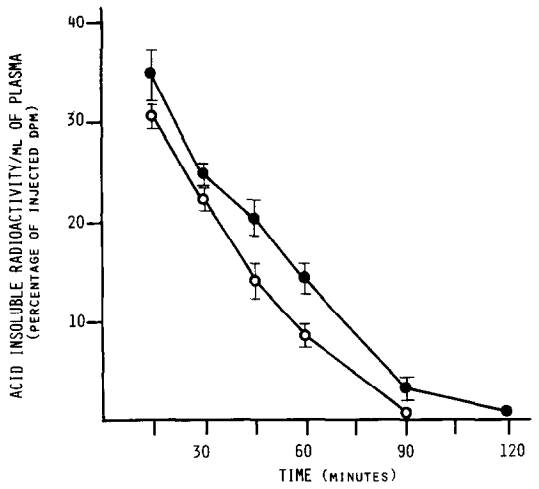

Fig. 1. Acid insoluble radioactivities in plasma of mice injected i.v. with conjugates (52 μg/g) labelled in the albumin (●) or in the drug moiety (○). Each point represents the mean value of results from four mice. Vertical bars denote S.E.

tetramer and heavier polymers in a conjugate preparation were 37, 22, 15, 8 and 18 respectively. These percentages did not change with time in contrast to the conjugate prepared at acidic pH, in which a further polymerization takes place after lyophilization [23].

Stability of the bond(s) between ara-AMP and L_{30}*-HSA in blood*

The levels of radioactivity in the acid insoluble fraction of mouse plasma after injection of the conjugate labelled in the ara-AMP moiety were only slightly lower than those observed after administration of an equal dose of L_{30}-[^{3}H]HSA-ara-AMP_{14}, in which the radioactive label was linked to protein by a bond which is very strong and not enzymatically cleaved [30] (Fig. 1). This indicates that most of the coupled ara-AMP molecules remained linked to L_{30}-HSA in mouse plasma.

Table 1. Effect of L_{30}-HSA-ara-AMP_{14} on plasma disappearance of [^{14}C]AF

Compound injected with [^{14}C]AF	Plasma (dpm/ml)
None	3658 ± 621
HSA	3520 ± 501
AF	9106 ± 1,022
L_{30}-HSA	9470 ± 1,370
L_{30}-HSA-ara-AMP_{14}	11,185 ± 1,105

Fetuin was enzymatically desialylated [33]. AF was labelled with [^{14}C]formaldehyde according to [34]. Female Swiss mice weighing 28/30 g were injected i.v. with 2 μg/g [^{14}C] AF (4.9×10^6 dpm/mg). HSA, AF, L_{30}-HSA, and the conjugate were administered i.v. simultaneously with [^{14}C]AF at 2 μg/g body weight. In all cases the volume injected was 10 μl/g. After 10 min animals were bled from retroorbital plexus under ether anaesthesia and the radioactivity of plasma was measured. Each entry represents the mean value ± S.E. of results from 10 animals.

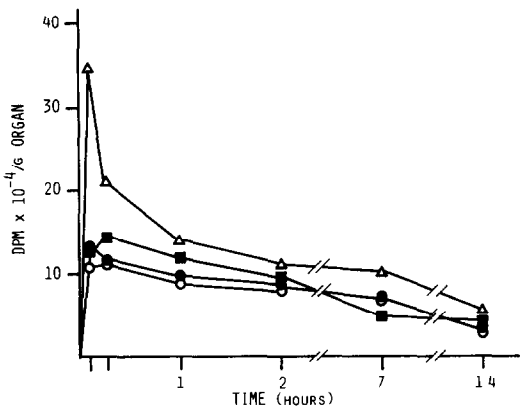

Fig. 2. Organ distribution of radioactivity in mouse after i.v. injection of free ara-[2,8-^{3}H]AMP (7.5 μg/g) (spec. act. 1.4×10^4 dpm/μg). Key: liver (●); kidneys (△); spleen (■); intestine (○). The experiments were carried out according to Fiume *et al.* [35]. Each entry represents the mean value of results from four animals. S.E. ranged from 2 to 12% of mean values.

Interaction of the conjugate with hepatocyte receptor

The disappearance of [^{14}C]-labelled AF from the blood of mice was competitively inhibited to the same extent by the conjugate and by an equal amount of non conjugated L_{30}-HSA (Table 1). This indicates that conjugation did not change the capacity of L_{30}-HSA to interact with the receptor for galactosyl terminating glycoproteins on the surface of hepatocytes.

Organ distribution of radioactivity after injection of free and coupled ara-[2,8-^{3}*H*]*AMP*

Free ara-[2,8-^{3}H]AMP was injected i.v. at a dose of 7.5 μg/g which corresponded to that administered to patients with chronic hepatitis B (5–10 mg/kg/day) [15]. Coupled ara-[2,8-^{3}H]AMP was injected i.v. at the smaller doses of 2 or 3 μg/g which corresponded to 35 or 52 μg conjugate respectively. Both free and coupled drug had the same specific activity (1.4×10^4 dpm/μg). In mice injected with the free drug, acid soluble radioactivity was equally distributed in liver, spleen and intestine; higher values were found in kidneys (Fig. 2). In mice administered with the conjugate, the values of both acid soluble and insoluble radioactivities were much higher in liver than in the other organs (Fig. 3). Moreover in these animals the liver levels of acid soluble radioactivity, which was due to the drug released from L_{30}-HSA, were higher than those of mice administered with free drug. The contrary was observed in the other organs. The sustained amount of acid soluble radioactivity detected in liver up to 15 hr after administration of conjugate indicates that ara-AMP (and/or its metabolites) remains, in large part, inside the hepatocytes after release from the carrier.

Effect of free and coupled ara-AMP on thymidine incorporation into DNA in liver, intestine and bone marrow of mice with Ectromelia virus hepatitis

In mice with Ectromelia virus hepatitis thymidine incorporation in liver is likely to be due to virus

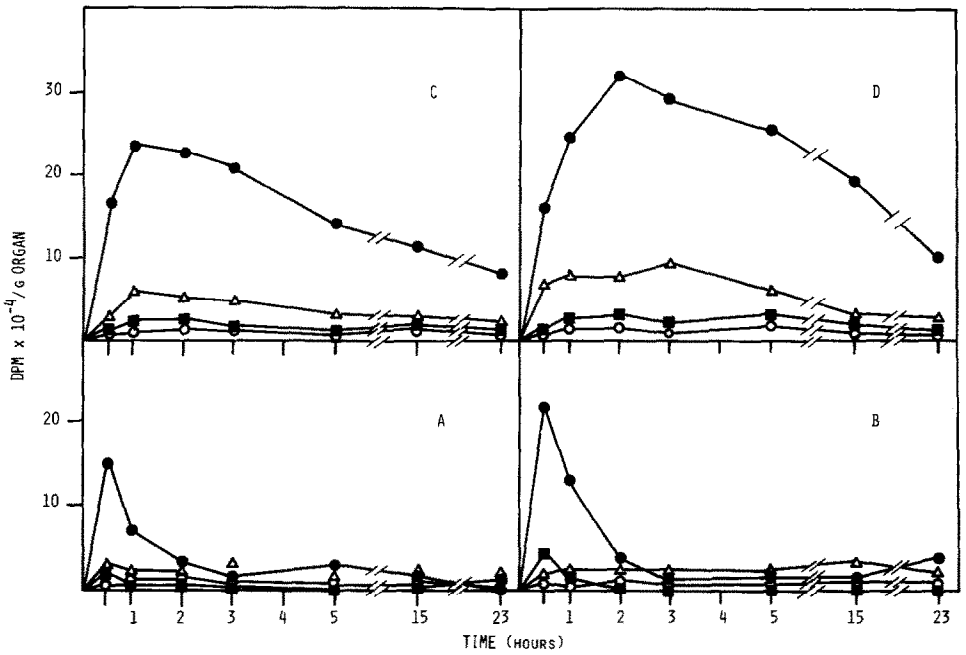

Fig. 3. Organ distribution of acid insoluble (frames A and B) and soluble (frames C and D) radioactivities after injection of coupled ara-[2,8^{3}H]AMP (spec. act. 1.4 × 10^4 dpm/μg) at the doses of 2 μg/g (frames A and C) or 3 μg/g (frames B and D). Symbols are the same as in Fig. 2. The experiments were performed as previously described [35]. The acid insoluble radioactivity was obtained by subtracting acid soluble from total radioactivity. Each entry represents the mean value of results from four animals. S.E. ranged from 3 to 21% of mean values.

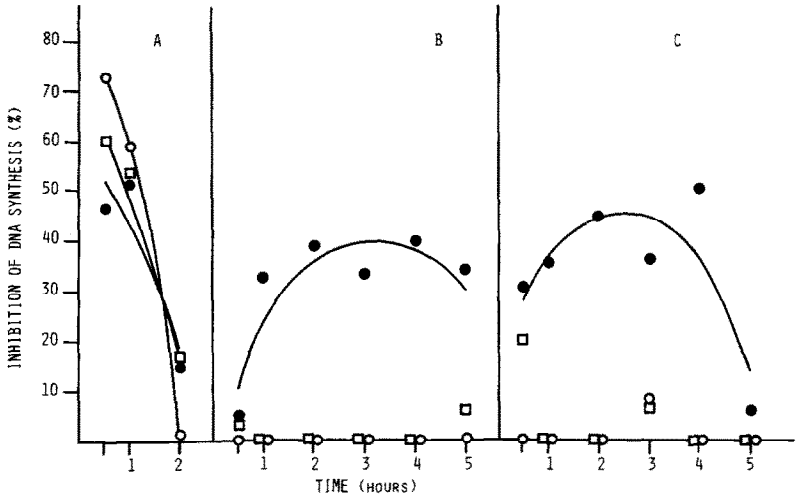

Fig. 4. Effect of free and coupled ara-AMP on [methyl-^{3}H]thymidine incorporation into DNA in liver (●), intestine (○) and bone marrow (□) of Ectromelia virus infected mice. Free ara-AMP (frame A) was injected i.v. at a dose of 7.5 μg/g; coupled ara-AMP was injected at doses of 2 μg/g (frame B) or 3 μg/g (frame C). The experiments were carried out as previously described [4]. Mice were killed 30 min after an i.p. injection of [methyl-^{3}H]thymidine (spec. act. 25 Ci/mmol) (0.7 μCi/g body weight). In control animals the mean values of dpm/100 μg DNA ranged in the different experiments from 23,649 to 31,120 in liver, from 43,474 to 52,340 in intestine and from 19,284 to 24,331 in bone marrow. Data have been evaluated by means of the analysis of variances. When the interaction time by treatment was found to be significant the regression lines have been calculated by the method of least squares. For each time the percentages of inhibition experimentally determined are indicated by the symbols. Coupled ara-AMP was never found to cause a significant inhibition in intestine or in bone marrow as evaluated by Student's t-test.

DNA synthesis [3]. Free ara-AMP (7.5 μg/g) inhibited thymidine incorporation in liver, intestine and bone marrow at 30 min and 1 hr. At 2 hr there was no longer significant inhibition in any of the three organs (Fig. 4). Coupled ara-AMP (2 or 3 μg/g) inhibited DNA synthesis in liver from 1 to 4 hr without producing significant inhibition in intestine and bone marrow at any time (Fig. 4). The inhibition produced in liver by coupled ara-AMP at the dose of 3 μg/g reached the same level (40–50%) as that caused in this organ by the free drug.

Experiments on conjugate acute toxicity

L_{30}-HSA-ara-AMP_{14} injected i.v. at 1.38 mg/g body weight did not display any recognizable sign of toxicity. For 2–3 hr after conjugate injection, the mice appeared less active compared to untreated animals but the same response was observed in mice injected with saline alone. Afterwards mice injected with conjugate behaved completely normally. The increase in the body weight as well as the food intake/day was equal in the three groups of animals (injected with conjugate, with saline alone or untreated). The highest dose tested was 1.38 mg/g and is about forty times higher than that effective in inhibiting virus DNA synthesis in liver of Ectromelia virus infected mice.

Immunogenicity of conjugates prepared with homologous or heterologous lactosaminated albumin

In not one of the 15 mice treated with L_{30}-MSA-ara-AMP_{14} were specific antibodies found in the serum. Antibodies against L_{30}-MSA-ara-AMP_{14} were produced in 3 out of 4 rats immunized with this conjugate. The antigen binding capacities of the sera were 0.45, 1.1 and 1.2 nmoles conjugate/ml serum which corresponded to about 34, 83 and 90 μg IgG/ml respectively. Antibodies binding L_{30}-HSA-ara-AMP_{14} were found in 14 out of 15 mice immunized with this conjugate. The antigen binding capacities of sera ranged from 0.2 to 1.2 nmoles of conjugate/ml (mean value 0.6 nmoles) which corresponded to about 15–90 μg IgG/ml (mean value 45 μg/ml).

DISCUSSION

In the treatment of chronic hepatitis B, ara-A and ara-AMP can be administered only in small doses and for relatively short periods because of the severe side effects [5–10]. These drugs are also toxic for lymphocytes [6]. After a few days administration they produce marked lymphocyte fragility and lymphocytopenia [6, 9] which may affect the outcome of the treatment since successful antiviral therapy appears to require the cooperation of the host immune system [6]. A selective delivery of ara-AMP to liver should result in a more efficient inhibition of virus replication accompanied by a lower toxicity for the other tissues including lymphocytes.

These effects should permit prolongation of the cycles of antiviral treatment and improve the cooperation of the immune response. The new ara-AMP conjugate with lactosaminated albumin fulfils the criteria required for a lysosomotropic drug–carrier complex [32] and possesses the properties to accomplish liver targeting of ara-AMP. It is quite stable in the bloodstream where only a small part of drug is released; it maintains the capacity of lactosaminated albumin to interact with the specific receptors on the surface of hepatocytes with which it can freely come in contact since hepatic sinusoids are not a barrier for proteins. The conjugate is selectively taken up by the liver where the albumin carrier is digested. The released drug remains in high concentration in liver and its pharmacological action is confined to this organ. Moreover, the conjugate does not display any recognizable sign of acute toxicity even at doses several fold higher than those pharmacologically active and, when prepared with homologous albumin, is not immunogenic. Finally a great practical advantage of this conjugate is its chemical stability; in contrast to those previously used [3, 4] it remains soluble after preparation. This characteristic should allow clinical testing of its efficiency in the treatment of chronic hepatitis B virus infection.

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