Biochemical Pharmacology, Vol. 54, pp. 357–363, 1997.

ISSN 0006-2952/97/$17.00 + 0.00
PII S0006-2952(97)00223-2

Ribavirin Conjugated with Lactosaminated Poly-L-lysine

SELECTIVE DELIVERY TO THE LIVER AND INCREASED ANTIVIRAL ACTIVITY IN MICE WITH VIRAL HEPATITIS

Giuseppina Di Stefano, Francesco P. Colonna,† Alessandro Bongini,‡ Corrado Busi,* Alessandro Mattioli* and Luigi Fiume*§*

*Dipartimento di Patologia sperimentale, Università di Bologna, 40126 Bologna, Italy; †Istituto I.Co.C.E.A. CNR, 40129 Bologna, Italy; and ‡Dipartimento di Chimica G. Ciamician, Università di Bologna, 40126 Bologna, Italy

ABSTRACT. Ribavirin (RIBV) is a useful drug in the treatment of chronic type C hepatitis but displays a toxicity for red blood cells (RBC), which limits its dosage and necessitates withdrawal in some patients. Selective concentration of RIBV in liver should improve therapeutic results. Liver targeting can be achieved by coupling the drug to galactosyl-terminating peptides, which specifically enter hepatocytes. In the present work, we conjugated RIBV to lactosaminated poly-L-lysine (L-Poly(Lys)), a hepatotropic carrier enabling intramuscular (IM) administration of conjugates. The L-Poly(Lys)-RIBV conjugate had a heavy drug load (312–327 μg of RIBV in 1 mg of conjugate) and was very soluble in 0.9% NaCl (200 mg/mL). The conjugate was devoid of acute toxicity in mouse. When incubated with human or mouse blood, it did not release the drug. After IM administration to mice, the conjugate was selectively taken up by the liver, where the drug was released in a pharmacologically active form. This was demonstrated using mice infected with a strain of murine hepatitis virus (MHV) sensitive to RIBV. Coupled RIBV, IM injected, inhibited MHV replication in liver at a daily dose two to three times lower than that of the free drug. In mice IM injected with a conjugate tritiated in the RIBV moiety, the ratios between the levels of radioactivity in liver and RBC were two times higher than in animals injected with free tritiated RIBV. In conclusion, the present results support the possibility that the chemotherapeutic index of RIBV in chronic type C hepatitis can be increased by conjugation with L-Poly(Lys). BIOCHEM PHARMACOL **54**;3:357–363, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. antiviral chemotherapy; drug targeting; lactosaminated poly-L-lysine; asialoglycoprotein receptor; ribavirin; hepatitis C

1-β-D-Ribofuranosyl-1,2,4-triazole-3-carboxamide (ribavirin) (RIBV[‖]) [1] (Fig. 1) is at present the only synthetic drug found to be useful in the treatment of chronic type C hepatitis [2–4]. Administered in association with α-interferon, it caused a sustained normalisation of liver transaminases and loss of serum hepatitis C virus RNA in a significantly higher number of patients than in the group treated with interferon alone [4]. However, RIBV accumulates in red blood cells (RBC), causing hemolysis [2, 4]. This side effect hinders administration of oral doses higher than 800–1,200 mg/day and necessitates withdrawal of the drug in some patients. Selective delivery of RIBV to the liver should reduce the risk of anemia and permit higher hepatocyte drug concentrations with improved therapeutic results. Such liver targeting can be obtained by conjugation with galactosyl-terminating peptides [5–8], which specifically penetrate parenchymal liver cells where they are digested in lysosomes [9]. This chemotherapeutic strategy has been supported by clinical studies. In patients with chronic B virus hepatitis, adenine arabinoside monophosphate (ara-AMP) conjugated with lactosaminated human albumin (L-HSA) inhibited virus replication when given at a daily dose three to six times lower than the free drug [10, 11]. L-HSA-ara-AMP, administered for 28 days, exerted antiviral activity to the same extent as the free drug without producing any clinical side effects, including the severe neurotoxicity caused by free ara-AMP [12].

We describe here the preparation and characterization of an RIBV conjugate with lactosaminated poly-L-lysine (L-Poly(Lys)), a carrier that enables conjugate administration by the intramuscular (IM) route [13–16]. We measured

§ Corresponding author: Dr. L. Fiume, Dipartimento di Patologia sperimentale, Via S. Giacomo 14, 40126 Bologna, Italy. Tel. 51-354721 or 51-354700; FAX 51-354746.

‖ *Abbreviations:* ALT, alanine aminotransferase; ara-AMP, adenine arabinoside monophosphate; FCS, fetal calf serum; IM, intramuscular route; L-HSA, lactosaminated human serum albumin; L-HSA-ara-AMP, conjugate of L-HSA with ara-AMP; MEM, minimum essential medium; MHV, mouse hepatitis virus; Poly(Lys), poly-L-lysine; L-Poly(Lys), lactosaminated Poly(Lys); L-Poly(Lys)-A, conjugate of L-Poly(Lys) with adenosine; L-Poly(Lys) L-RIBV; γ, conjugate of L-Poly(Lys) with ribavirin; RBC, red blood cells; RIBV, ribavirin; RIBVMP, RIBV monophosphate.

Received 20 September 1996; accepted 13 December 1996.

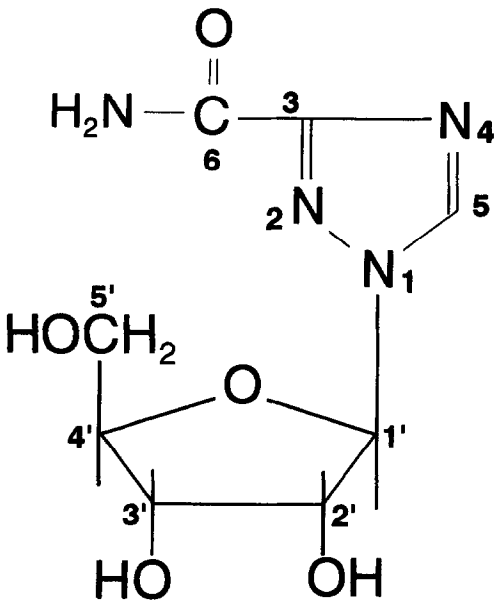

FIG. 1. Ribavirin (RIBV).

the organ distribution of this conjugate labeled in the lactose moiety and IM injected to mice. Moreover, we determined the levels of radioactivity in liver and RBC of mice IM injected with free and L-Poly(Lys)-coupled tritiated RIBV. Finally, we studied the antiviral activity of the free and coupled drug in liver of mice infected with a strain of mouse hepatitis virus (MHV) sensitive to RIBV [17].

MATERIALS AND METHODS

Preparation of the Conjugate

Coupling of lactose was obtained by reductive amination [18]. In a typical preparation, 200 mg of Poly(Lys) · HBr (Sigma Chemical Co., St Louis, MO, USA) with a molecular mass ranging from 11,000 to 32,000 Da was dissolved in 20 mL of water together with 800 mg α-lactose and 500 mg of $NaBH_3CN$. After incubation at 37° for 3 h, the solution was diafiltered with 0.9% NaCl using a 3000-Da cut-off membrane (Amicon, Inc., Beverly, MA, USA) and concentrated to 100 mg/mL. Lactose content was measured according to Dubois *et al.* [19] using galactose as a standard. Poly(Lys) was measured by reading the absorbance at 235 nm. This wavelength was chosen to avoid bromide interference. The lactose content ranged from 430 to 460 μg/mg L-Poly(Lys) in the different preparations. ^{14}C-Labeled L-Poly(Lys) was obtained by using [D-glucose-1-^{14}C]lactose (Amersham International, Buckinghamshire, U.K.). It contained 460 μg of lactose/mg and had a specific activity of 1.3×10^6 dpm/mg.

Conjugation of RIBV (Alfa Wassermann, Bologna, Italy) was obtained via the imidazolide of 5'-monophosphate of RIBV (RIBVMP) [20]. RIBV was phosphorylated in its primary OH group according to Yoshikawa *et al.* [21] and purified using a column of activated charcoal. RIBVMP, as pyridinium salt, was converted to imidazolide according to Lohrmann and Orgel [22]. In a typical conjugate preparation, 4 mL of L-Poly(Lys) (400 mg) was diluted with 4 mL of 1 M sodium carbonate buffer, pH 9.5; 1.6 g of RIBVMP imidazolide were added, and the pH was readjusted to 9.5 with NaOH. After incubation at 37° for 96 h, the solution was diafiltered with 0.15 M NaCl using a 3,000-Da cut-off membrane, and the pH was adjusted to 7. The RIBV content of the conjugate was measured by determining the organic phosphate (linking the drug to L-Poly(Lys)) according to Ames [23]. Lactose content was measured according to Dubois *et al.* [19] using galactose as a standard. The interference of the ribose moiety of RIBV in the colorimetric assay was calculated and subtracted. Care was taken to prepare the standards of both galactose and RIBV with sugar contents similar to those of the sample to be tested. The accuracy of this procedure was evaluated using ^{14}C-labeled L-Poly(Lys)-RIBV. The amount of lactose determined by radioactivity counting exhibited only a 1–3% difference from that calculated with the colorimetric method. The Poly(Lys) content of the conjugate was calculated from the amount of lactose, the lactose/Poly(Lys) weight ratio having been determined before drug coupling. The conjugate was sterilized by passing through a 0.45-μm filter and concentrated to 200 mg/mL. Radioactive conjugates with [^{14}C]lactose or [^{3}H]RIBV (see below) were prepared using the same percentages of reactants on a small scale.

To prepare a conjugate labelled in the drug moiety, the commercially available radioactive RIBV (random-tritiated) could not be used because tritium was completely lost during the coupling procedure. Therefore, we introduced tritium into the RIBV molecule with a stable bond following the approach by Baker and Haskell [24]. These authors first oxidized the primary OH group of the sugar moiety of adenine arabinoside and then reduced the nearly formed aldehyde with tritiated borohydride. The [^{3}H]RIBV preparation procedure will be described in detail elsewhere. The chemical structure of [^{3}H]RIBV was confirmed by the ^{13}C NMR spectrum, which showed the resonances of the parent compound. Tritiated RIBV was 98% pure, assessed by high performance liquid chromatography, and had a specific activity of 49,000 dpm/μg.

Biological Studies

Female Swiss and Balb/C mice were purchased from Harlan Nossan (Milan, Italy) and were maintained in an animal facility at the Department of Experimental Pathology of Bologna. They received humane care in accordance with the guidelines of the Italian Ministry of Health. Intramuscular injections were performed into the back muscles of the hind legs. Compounds were injected in a volume of 10 μL using a 25-μL microsyringe.

The study of the antiviral activity of L-poly(Lys)-RIBV was performed using the Friend-Braunsteiner strain of MHV, which was found to be sensitive to RIBV by Sidwell *et al.* [17]. The virus was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA.) and

was twice passed into suckling Swiss mice weighing 8–10 g. After 48 h, mice were killed at the first signs of suffering; livers were pooled and homogenized in 10 volumes of minimum essential medium (MEM) (Sigma) supplemented with 10% fetal calf serum (FCS) (Sigma). The suspension was clarified by centrifugation (5 min, 2,000 × g) and was subsequently sterilized through 0.45- and 0.22-μm syringe filters. Homogenates were frozen in separate portions in liquid nitrogen and stored at −80°. Each portion was thawed and used only once. Before the experiments commenced, a sample was titrated on NCTC clone 1469 cells as described below. NCTC clone 1469 cells were obtained from ATCC and were grown in NCTC 135 medium (Gibco BRL, Paisley, Scotland) supplemented with 10% horse serum (Gibco BRL).

Female 6-week-old Balb/C mice, weighing 16–18 g, specifically pathogen-free were inoculated intraperitoneally with approximately 100 CLD_{50} (lethal dose for 50% cultures in our titration assay). The compounds, free RIBV, coupled RIBV, and a control L-Poly(Lys) conjugate of adenosine (L-Poly(Lys)-A, prepared as described previously [15]) or saline were IM administered according to the schedule of Sidwell *et al.* [17] (twice daily beginning 2 h before virus inoculation). Five animals were used in each treatment group. Mice were killed when showing the first signs of suffering (48 h after virus inoculation in experiments 1 and 2; 72 h in experiment 3 of Table 3). Liver homogenates were prepared as described above, but without FCS. In experiment 1, the livers of each treatment group were pooled, whereas in experiments 2 and 3 liver samples were partly pooled and partly kept separate. Virus titration was performed first on pooled livers of each group; when a difference of *ca.* 1 log between virus titers of two groups was found, the titration was repeated on the separate liver samples to assess the significance of the difference according to Student's *t* test. Titration was performed on monolayers of NCTC clone 1469 cells grown in 96-well Falcon Microtest III tissue culture plates (Becton Dickinson, Lincoln Park, N. J., USA). Liver homogenates were diluted with MEM 10-fold through 10^{-8}, and 50 μL were added to the cell monolayer. Six wells were prepared for each dilution. After 2 h, the inoculum was removed and the cultures were maintained for 3 days or more in NCTC 135 medium supplemented with 2% chicken serum (Sigma). The viral cytopathic effect (complete destruction of the cell monolayer) was determined after 72 h. In cultures surviving this time, no cytopathic effect appeared in the following days. 50% endpoints were determined by the method of Reed and Muench [25].

RESULTS

Chemical Characteristics of Conjugate

The first attempts at conjugating RIBV were performed by phosphorylating the drug in the 5′ position of the sugar and allowing the resulting compound to react with L-Poly(Lys) in the presence of 1-ethyl-3-(dimethyl aminopropyl)carbodiimide. Although the reaction conditions were those successfully used for preparing some L-Poly(Lys)-ara-AMP conjugates [8, 13, 26], the preparations of L-Poly(Lys)-RIBV had a very low weight ratio of drug/carrier. Therefore, coupling was performed via the imidazolide [20] of the phosphate ester of RIBV (RIBVMP). Since this procedure requires high pH values [14, 20] that can modify the RIBV molecule, we first studied the percentages of RIBV and RIBVMP recovered after incubation under different alkaline conditions. When kept at pH 11 and 50° for 96 h (the conditions used for coupling ara-AMP to L-Poly(Lys) via its imidazolide [14–16]), both RIBV and RIBVMP were transformed in large part into compounds that behaved as more acidic molecules; in fact, when chromatographed on an HPLC anion exchanger column, their retention times were higher than those of the parent compounds. The new molecules probably resulted from hydrolysis of the carboamide group (Fig. 1) forming carboxylic derivatives. This was confirmed by the ^{13}C NMR spectrum of the modified RIBV. Compared to that of RIBV, it showed a change in

TABLE 1. Carbon-13 NMR chemical shifts (in ppm) of RIBV, RIBV derivative, RIBVMP, and conjugated RIBV

RIBV	RIBV derivative*	RIBVMP	L-Poly(Lys)-RIBV†	Assignment
157.2	160.4	157.0	157.2	C3
146.9	146.3	146.6	146.9	C5
163.5	166.8	163.6	163.3	C6
92.3	92.0	92.8	92.7	C1′
75.3	75.2	75.6	75.4	C2′
70.8	71.0	71.1	71.2	C3′
85.8	85.8	85.5 (J_{PC} = 8.4 Hz)	85.0 (J_{PC} = 8.7 Hz)	C4′
61.9	62.2	64.1 (J_{PC} = 4.8 Hz)	64.8 (J_{PC} = 3.6 Hz)	C5′

^{13}C NMR spectra were recorded at 75 MHz with a Varian Gemini 300 instrument, flip angle 60°, transient number 1,000–50,000, temperature 20°. The samples were dissolved in D_2O (20 mg/mL), and dioxane in D_2O (d = 67.4) was used as external reference.

* RIBV was kept at pH 11, 50° for 148 h. It was completely transformed into the derivative that displayed an increased retention time when chromatographed on an anion exchanger column (see "Results").

† In L-Poly(Lys)-RIBV prepared using the coupling conditions employed for obtaining the similar conjugate with ara-AMP [14], RIBV C6 showed a second peak with the value of 166.8 ppm (see text).

the resonance of the carbon 6 of this group (from 163.5 to 166.8 ppm) (Table 1).

On the basis of the finding that neither RIBV nor RIBVMP was changed when incubated at pH 9.5 and 37° for 96 h, we performed the conjugation under these conditions (see "Materials and Methods").

In the ^{13}C NMR spectrum of the L-Poly(Lys)-RIBV conjugate, the resonances attributable to RIBV were detected (Table 1). The peak corresponding to carbon 6 of the derivative formed under strong alkaline conditions (ppm 166.8) (see above) was not observed. These findings indicate that most of the RIBV molecules were not changed by the present conjugation procedure. In the ^{13}C NMR spectrum of a conjugate obtained by incubating RIBVMP imidazolide under the incubation conditions used for preparing the ara-AMP conjugate [14], carbon 6 showed two peaks with the values of 163.5 and 166.8 ppm, respectively. The ratio of intensities of the two peaks was 2:1 (data not shown in Table 1). This confirmed that a part of the RIBV molecules was modified by the drastic conditions of the synthesis of the ara-AMP conjugate.

The contents of Poly(Lys), lactose, and RIBV in 1-mg samples of conjugate ranged between narrow limits among the different preparations; they were 286–322, 238–260, and 312–327 μg, respectively. Considering that 1 mg of Poly(Lys) contains 7.8 μmol of lysine residues and that the molecular weights of lactose and RIBV are 342 and 244.2, respectively, it can be calculated that the ϵ-NH_2 groups of Poly(Lys) were substituted by the sugar and by the drug in the percentage ranges of 27–30 and 54–57, respectively.

The conjugate was soluble in saline (0.9% NaCl) at 200 mg/mL. The concentrated solution was stable when maintained either frozen or at 0–4°; after 6 months the weight ratio of RIBV/L-Poly(Lys) was not changed. The conjugate was not kept in a lyophilized form because after freeze-drying it can not be redissolved at 200 mg/mL.

Biological Properties of Conjugate

No clinical signs or behavioural alterations were observed in any of the five female Swiss mice (28–30 g), which received a single i.v. injection of the conjugate at the dose of 2 mg/g. The body weight gain, measured 3, 7, and 14 days after conjugate injection, was similar in treated mice and controls i.v. injected with saline.

The stability of the bond between RIBV and L-Poly(Lys) in blood was studied using L-Poly(Lys)-[^{3}H]RIBV, following the procedure described by Di Stefano *et al.* [14]. L-Poly(Lys)-[^{3}H]RIBV incubated in mouse or human blood at 37° for up to 6 h did not release the drug.

Fig. 2 shows that after IM administration to mice, ^{14}C-labeled L-Poly(Lys)-RIBV was selectively taken up by the liver. The values of radioactivity in kidney were low, indicating that this conjugate was lost through the kidney only in small quantities.

The antiviral activity of the conjugate was studied in Balb/C mice with hepatitis caused by MHV (Friend-

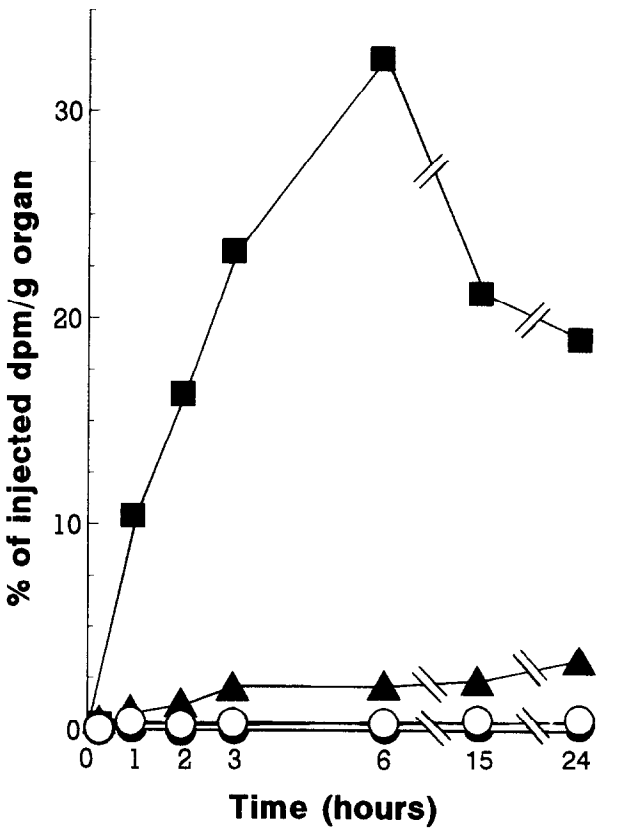

FIG. 2. Distribution of radioactivity in liver (■), kidney (▲), spleen (●), and intestine (○) of female Swiss mice (28–30 g) injected IM with ^{14}C-labeled L-Poly(Lys)-RIBV (6.5 μg/g; specific activity, 750 dpm/μg). The experimental procedure was described previously [15]. Since the conjugate was not precipitated by either trichloroacetic or perchloric acid, only total (acid soluble + insoluble) radioactivity was measured. Each entry represents the mean value of results from two animals. SE ranged from 1% to 7% of mean values.

Braunsteiner strain). This virus, which replicates in mouse hepatocytes and causes liver necrosis, has been found to be sensitive to RIBV [17]. Table 2 shows the effect of IM administration of free and conjugated RIBV on virus replication in liver and on serum alanine aminotransferase (ALT) levels. Free RIBV at the daily dose of 20 μg/g had no effect on virus titer; at the daily dose of 30 μg/g it inhibited virus replication in experiment 2 but had no significant effect in experiment 3. Conversely, at 30 μg/g the free drug significantly decreased serum ALT levels in experiment 3 but was ineffective on this parameter in experiment 2. Coupled RIBV inhibited virus replication in liver and significantly decreased serum ALT levels at the daily dose of only 13.2 μg/g. The conjugate with adenosine (L-Poly(Lys)-A) had no significant effects either on virus titer or on serum ALT levels. This control conjugate was used to verify that the antiviral activity of L-Poly(Lys)-RIBV was caused by the RIBV moiety. In conclusion, the experiments using MHV-infected mice indicated that in liver the conjugate released the drug in a pharmacologically active form and that the coupled drug exerted the antiviral

TABLE 2. Effect of free and coupled RIBV on virus replication in liver and on ALT levels in serum of Balb/C mice infected with MHV

Experiment	Compound	Daily dose of RIBV (μg/g)*	Liver virus titer (log_{10})†	Serum ALT levels (U/l)‡
1	Saline	0	5.5	
	Free RIBV	20	5.8	
	Coupled RIBV	20 (66.6)§	4.6	
2	Saline	0	4.8 ± 0.4‖	683.1 ± 243.7‖
	Free RIBV	30	3.8 ± 0.1 (p = 0.042)	191.8 ± 112.0 (NS)
	Coupled RIBV	13.2 (44)	3.6 ± 0.2 (p = 0.028)	64.9 ± 19.8 (p = 0.035)
3	Saline	0	6.6 ± 0.3	6067.3 ± 1434.2
	Free RIBV	30	6.8	1203.3 ± 399.8 (p = 0.008)
	Coupled RIBV	13.2 (44)	5.1 ± 0.2 (p = 0.003)	722.8 ± 325.3 (p = 0.005)
	L-Poly(Lys)-A¶	0	6.2	2840.2 ± 860.8 (NS)

* Compounds were IM injected twice daily beginning 2 h before virus inoculation. Mice were killed at first signs of suffering (48 h in experiments 1 and 2; 72 h in experiment 3).

† Reciprocal of liver homogenate dilution killing 50% cultures of NCTC 1469 cells (see "Materials and Methods").

‡ Serum ALT activity was determined colorimetrically using the kit commercially available from Boehringer (Mannheim, Germany). The normal values of serum ALT activity in noninfected Balb/C mice were 51.6 ± 5.2.

§ In parentheses the amount (μg/g) of conjugate containing the administered dose of RIBV.

‖ Standard error.

¶ This conjugate was administered at the daily dose of 43 μg/g.

activity at a daily dose two to three times lower than that of the free RIBV.

Since the main toxic effect of RIBV is hemolysis caused by drug accumulation in RBC [27, 28], we studied the penetration of free and coupled [^{3}H]RIBV into human and mouse erythrocytes incubated *in vitro*. Moreover, we measured the levels of radioactivity in liver and RBC of mice IM administered with free and conjugated radioactive drug. The results of *in vitro* experiments are shown in Fig. 3. Free [^{3}H]RIBV entered in erythrocytes in large amounts, in agreement with the observation that nucleosides and their analogs, including RIBV [27, 28], are rapidly transported inside RBC. On the contrary the conjugate did not penetrate in RBC. This can be easily explained since erythrocytes do not display endocytotic activity. Table 3 shows the levels of radioactivity and the percentages of injected dpm in liver and RBC of mice after IM administration of free or coupled [^{3}H]RIBV injected at the dose of 2 or 7 μg/g. The second dose was approximately that which inhibited MHV replication in liver in mice injected with the conjugate, when given twice a day (see above). At the same dose of free and coupled radioactive drug and at the same interval after injection, the ratios between dpm in liver and those in red blood cells were two times higher in mice administered with the conjugate.

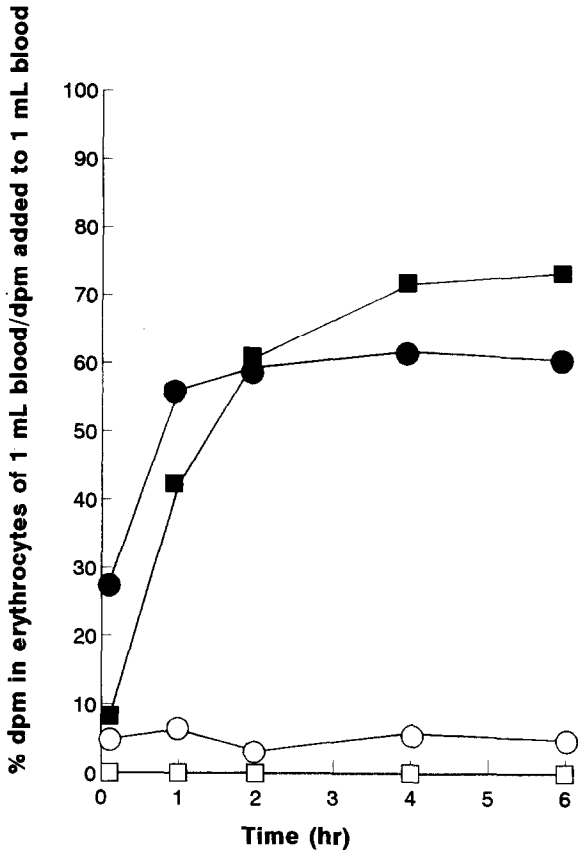

FIG. 3. Radioactivity in human (■, □) and mouse (●, ○) RBC when the blood was incubated in the presence of free [^{3}H]RIBV (■, ●) or L-Poly(Lys)-[^{3}H]RIBV (□, ○). Blood was incubated at 37° as described previously [31]. Free and coupled [^{3}H]RIBV were added at the concentration of 10 μg/mL (10 μg of coupled [^{3}H]RIBV] corresponds to 31.7 μg of conjugate). At different times, samples of blood were centrifuged at 1,500 g for 5 min, and the concentration of radioactivity (dpm/mL) in RBC was measured as described [27]. Each entry represents the mean value of results from two experiments. Standard errors ranged from 0% to 4.8% of mean value.

DISCUSSION

We conjugated RIBV to L-Poly(Lys) to obtain a liver targeting of this drug and thereby reduce its toxic effects on erythrocytes in the treatment of chronic hepatitis C. The procedure of synthesis adopted allowed the preparation of a very soluble conjugate with a heavy RIBV load. These properties are required for administering the conjugate in a small volume. The substitution of a high number of ε-amino groups is also necessary to eliminate the toxicity and immunogeniticy of Poly(Lys) [14–16].

TABLE 3. Levels of radioactivity in liver and RBC of mice IM injected with free or coupled [^{3}H]RIBV

Compound	Dose (μg/g)	Time (h)	dpm × 10^{-3}*		dpm in liver
			Liver	RBC	dpm in RBC
Free [^{3}H]RIBV	2	0.5	439 ± 49 (14.9)†	21.0 (0.71)†	20.9
Coupled [^{3}H]RIBV	2	0.5	252 ± 83 (8.6)	6.9 (0.23)	36.5
Free [^{3}H]RIBV	2	1	439 ± 22 (14.9)	20.6 (0.70)	21.3
Coupled [^{3}H]RIBV	2	1	546 ± 60 (18.6)	8.6 (0.29)	63.5
Free [^{3}H]RIBV	2	2	305 ± 7 (10.3)	14.4 (0.49)	21.2
Coupled [^{3}H]RIBV	2	2	527 ± 85 (17.9)	10.6 (0.36)	49.7
Free [^{3}H]RIBV	2	4	210 ± 19 (7.6)	18.6 (0.67)	11.3
Coupled [^{3}H]RIBV	2	4	502 ± 16 (17.1)	15.6 (0.53)	32.2
Free [^{3}H]RIBV	7	1	1,832 ± 163 (17.1)	56.2 (0.54)	32.6
Coupled [^{3}H]RIBV	7	1	1,104 ± 3 (10.7)	18.3 (0.18)	60.3
Free [^{3}H]RIBV	7	2	1,070 ± 33 (10.4)	48.9 (0.47)	21.9
Coupled [^{3}H]RIBV	7	2	1,118 ± 68 (10.9)	23.5 (0.23)	47.6
Free [^{3}H]RIBV	7	4	625 ± 7 (6.1)	30.5 (0.29)	20.5
Coupled [^{3}H]RIBV	7	4	1,336 ± 38 (13.0)	34.4 (0.33)	38.8

Female Swiss mice of 28–30 g were used. Radioactivity in liver and red blood cells was measured as described by Fiume *et al.* [15] and by Carlin *et al.* [27], respectively. Each entry represents the mean value of results from two animals. Livers were processed separately and blood was pooled. Liver accumulation index (dpm in liver/dpm in RBC; % of administered radioactivity in liver/% of administered dpm in RBC) was statistically evaluated by a multivariate analysis of variance (MANOVA) using the RIBV form (free or coupled) as factor, doses and times as covariates. The effect of the drug form resulted to be remarkably significant in determining the radioactivity ratio yielding $p < 0.0005$, with observed statistical power >99% at 0.05 level.

* dpm are referred to 1 g of liver or to 1 mL of RBC.

† Percentages of injected radioactivity are indicated in parentheses.

L-Poly(Lys)-RIBV, IM administered to mice, was selectively taken up by the liver. In this organ the conjugate released the drug in a pharmacologically active form. This was demonstrated by the antiviral activity exerted by the conjugate in liver of mice with MHV hepatitis.

The conjugate incubated in human or mouse blood did not release the drug and did not enter into RBC. However, radioactivity was found in RBC of mice injected with the conjugate labeled in the drug moiety. This radioactivity was probably due to [^{3}H]RIBV and its metabolites [29], which partly went out from liver into the bloodstream after release of the drug from the carrier inside hepatocytes. A similar release of the drug from liver into bloodstream was observed in animals injected with trifluorothymidine-asialofetuin [5] or L-HSA-ara-AMP [30] conjugates.

In conclusion, coupled RIBV inhibited MHV replication in liver at a dose two to three times lower than the free drug, and the ratios between the levels of radioactivity in liver and RBC were twofold higher in animals injected with the conjugated drug. These findings support the possibility that the chemotherapeutic index of RIBV in treatment of chronic hepatitis C can be increased by conjugation with L-Poly(Lys).

At present the only tolerability study performed using L-Poly(Lys)-RIBV has been that of acute toxicity in mouse, which gave negative results. However, experiments with the similar complex L-Poly(Lys)-ara-AMP endorse the possibility of a clinical use of the RIBV conjugate. L-Poly(Lys)-ara-AMP was devoid of acute and subchronic (4 weeks) toxicity in mouse and rat [14–16] and when administered to mice by repeated IM injections did not produce antibodies [14].

For clinical use, the conjugate would have the disadvantages of the cost of synthesis and of administration by a parenteral route, compared to free RIBV. On the other hand, in patients where RIBV must be withdrawn because of anemia, the conjugate might be the appropriate form for administering this drug. Moreover, the conjugate might allow higher RIBV concentrations to be reached in liver and consequently, if administered in association with α-interferon, might increase the number of patients with sustained response.

This work was supported by grants from AIRC, the Istituto Superiore di Sanità (Progetto Epatite Virale), MURST, and the University of Bologna (funds for selected research topics).

References

1. Sidwell RW, Huffman JH, Khare GP, Allen LB, Witkowski JT and Robins RK, Broad-spectrum antiviral activity of virazole: 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide. *Science* **177:** 705–707, 1972.
2. Di Bisceglie AM, Shindo M, Fong TL, Fried MW, Swain MG, Bergasa NV, Axiotis CA, Waggoner JG, Park Y and Hoofnagle JH, A pilot study of ribavirin therapy for chronic hepatitis C. *Hepatology* **16:** 649–654, 1992.
3. Kakumu S, Yoshioka K, Wakita T, Ishikawa I, Takayanagi M and Higashi Y, A pilot study of ribavirin and interferon beta for the treatment of chronic hepatitis C. *Gastroenterology* **105:** 507–512, 1993.
4. Brillanti S, Garson J, Foli M, Whitby K, Deaville R, Masci C, Miglioli M and Barbara L, A pilot study of combination therapy with ribavirin plus interferon alfa for interferon alfa-resistant chronic hepatitis C. *Gastroenterology* **107:** 812–817, 1994.
5. Fiume L, Mattioli A, Balboni PG, Tognon M, Barbanti-Brodano G, De Vries J and Wieland T, Enhanced inhibition

of virus DNA synthesis in hepatocytes by trifluorothymidine coupled to asialofetuin. *FEBS Lett* **103:** 47–51, 1979.
6. Fiume L, Busi C, Mattioli A, Balboni PG and Barbanti-Brodano G, Hepatocyte targeting of adenine-9-β-D-arabinofuranoside 5′-monophosphate (ara-AMP) coupled to lactosaminated albumin. *FEBS Lett* **129:** 261–264, 1981.
7. Fiume L, Bassi B, Busi C, Mattioli A and Spinosa G, Drug targeting in antiviral chemotherapy: a chemically stable conjugate of 9-β-D-arabinofuranosyladenine 5′-monophosphate with lactosaminated albumin accomplishes a selective delivery of the drug to liver cells. *Biochem Pharmacol* **35:** 967–972, 1986.
8. Fiume L, Bassi B, Busi C, Mattioli A, Spinosa G and Faulstich H, Galactosylated poly(L-lysine) as a hepatotropic carrier of 9-β-D-arabinofuranosyladenine 5′-monophosphate. *FEBS Lett* **203:** 203–206, 1986.
9. Ashwell G and Harford J, Carbohydrate-specific receptors of the liver. *Annu Rev Biochem* **51:** 531–534, 1982.
10. Fiume L, Torrani Cerenzia MR, Bonino F, Busi C, Mattioli A, Brunetto MR, Chiaberge E and Verme G, Inhibition of hepatitis B virus replication by vidarabine monophosphate conjugated with lactosaminated serum albumin. *Lancet* **2:** 13–15, 1988.
11. Torrani Cerenzia MR, Fiume L, Busi C, Mattioli A, Di Stefano G, Gervasi GB, Brunetto MR, Piantino P, Verme G and Bonino F, Inhibition of hepatitis B virus replication by adenine arabinoside monophosphate coupled to lactosaminated albumin: efficacy and minimal effective dose. *J Hepatol* **20:** 307–309, 1994.
12. Torrani-Cerenzia MR, Fiume L, De Bernardi Venon W, Lavezzo B, Brunetto MR, Ponzetto A, Di Stefano G, Busi C, Mattioli A, Gervasi GB, Bonino F and Verme G, Adenine arabinoside monophosphate coupled to lactosaminated albumin, administered for 4 weeks to patients with chronic type B hepatitis, decreased viremia without producing significant side effects. *Hepatology* **23:** 657–661, 1996.
13. Fiume L, Di Stefano G, Busi C and Mattioli A, A conjugate of lactosaminated poly-L-lysine with adenine arabinoside monophosphate, administered to mice by intramuscular route, accomplishes a selective delivery of the drug to the liver. *Biochem Pharmacol* **47:** 643–650, 1994.
14. Di Stefano G, Busi C, Mattioli A and Fiume L, Selective delivery to the liver of antiviral nucleoside analogs coupled to a high molecular mass lactosaminated poly-L-lysine and administered to mice by intramuscular route. *Biochem Pharmacol* **49:** 1769–1775, 1995.
15. Fiume L, Di Stefano G, Busi C, Mattioli A, Rapicetta M, Giuseppetti R, Ciccaglione AR and Argentini C, Inhibition of woodchuck hepatitis virus replication by adenine arabinoside monophosphate coupled to lactosaminated poly-L-lysine and administered by intramuscular route. *Hepatology* **22:** 1072–1077, 1995.
16. Fiume L, Di Stefano G, Busi C, Mattioli A, Gervasi GB, Bertini M, Bartoli C, Catalani R, Caccia G, Farina C, Fissi A, Pieroni O, Giuseppetti R, D'Ugo E, Bruni R and Rapicetta M, The hepatotropic conjugate of adenine arabinoside monophosphate with lactosaminated poly-L-lysine. Synthesis of the carrier and pharmacological properties of the conjugate. *J Hepatol*, **26:** 253–259, 1997.
17. Sidwell RW, Huffman JH, Campbell N and Allen LB, Effect of ribavirin on viral hepatitis in laboratory animals. *Ann NY Acad Sci* **284:** 239–246, 1977.
18. Schwartz BA and Gray GR, Proteins containing reductively aminated disaccharides: synthesis and chemical characterization. *Arch Biochem Biophys* **181:** 542–549, 1977.
19. Dubois M, Gilles KA, Hamilton JK, Rebers PA and Smith F, Colorimetric method for determination of sugar and related substances. *Anal Chem* **28:** 350–356, 1956.
20. Fiume L, Busi C, Di Stefano G and Mattioli A, Coupling of antiviral nucleoside analogs to lactosaminated human albumin by using the imidazolides of their phosphoric esters. *Anal Biochem* **212:** 407–411, 1993.
21. Yoshikawa M, Kato T and Takenishi T, A novel method for phosphorylation of nucleosides to 5′-nucleotides. *Tetrahedron Lett* **50:** 5065–5068, 1967.
22. Lohrmann R and Orgel LE, Preferential formation of (2′-5′)-linked internucleotide bond in non-enzymatic reactions. *Tetrahedron* **34:** 853–855, 1978.
23. Ames BN, Assay of inorganic phosphate, total phosphate and phosphatases. *Methods Enzymol* **8:** 115–118, 1966.
24. Baker DC and Haskell TH, A labeled 9-β-D-arabinofuranosyladenine 5′-monophosphate having increased metabolic stability. *Ann NY Acad Sci* **284:** 30–33, 1977.
25. Reed LJ and Muench H, A simple method of estimating fifty per cent endpoints. *Am J Hyg* **27:** 493–497, 1938.
26. Fiume L, Bassi B and Bongini A, Conjugates of 9-β-D-arabinofuranosyladenine 5′-monophosphate (ara-AMP) with lactosaminated albumin: characterization of the drug-carrier bonds. *Pharm Acta Helv* **63:** 137–139, 1988.
27. Catlin DH, Smith RA and Samuels AI, ^{14}C-ribavirin: distribution and pharmacokinetic studies in rats, baboons and man. In: *Ribavirin a broad spectrum antiviral agent* (Eds. Smith RA and Kirkpatrick W), pp. 83–98. Academic Press, New York, 1980.
28. Laskin OL, Longstreth JA, Hart CC, Scavuzzo D, Kalman CM, Connor JD and Roberts RB, Ribavirin disposition in high-risk patients for acquired immunodeficiency syndrome. *Clin Pharmacol Ther* **41:** 546–555, 1987.
29. Miller JP, Kigwana LJ, Streeler DG, Robins RK, Simon LN and Roboz J, The relationship between the metabolism of ribavirin and its proposed mechanism of action. *Ann NY Acad Sci* **284:** 211–229, 1977.
30. Fiume L, Busi C, Corzani S, Di Stefano G, Gervasi GB and Mattioli A, Organ distribution of a conjugate of adenine arabinoside monophosphate with lactosaminated albumin in the rat. *J Hepatol* **20:** 681–682, 1994.
31. Zimmerman TP and Deeprose RD, Metabolism of 5-amino-1-β-D-ribofuranosyl-imidazole-4-carboxamide and related five-membered heterocycles to 5′-triphosphates in human blood and L5178Y cells. *Biochem Pharmacol* **27:** 709–716, 1978.