Biochemical Pharmacology, Vol. 59, pp. 301–304, 2000.

ISSN 0006-2952/00/$–see front matter
PII S0006-2952(99)00327-5

SHORT COMMUNICATION

Enhanced Liver Blood Concentrations of Adenine Arabinoside Accomplished by Lactosaminated Poly-L-lysine Coupling: Implications for Regional Chemotherapy of Hepatic Micrometastases

Giuseppina Di Stefano, Corrado Busi,* Alessandro Camerino,* Bruno Nardo† and Luigi Fiume*‡*

*Department of Experimental Pathology, University of Bologna, 40126 Bologna; and †Department of Transplants and Surgery, University of Bologna, 40138 Bologna, Italy

ABSTRACT. Conjugates of antiviral and antiblastic nucleoside analogs (NAs) with galactosyl-terminating peptides selectively enter hepatocytes after binding of the carrier galactose residues to the asialoglycoprotein receptor. Since NAs, when set free from the carrier within hepatocytes, partly exit from these cells into the bloodstream, we considered the possibility that administration of galactosyl-terminating conjugates of NAs could result in plasma concentrations of these drugs that would be higher in liver sinusoids than in capillaries of other organs. In the present study we demonstrated the validity of this hypothesis. We injected rats with a conjugate of adenine arabinoside (ara-A) with lactosaminated poly-L-lysine and found that the plasma concentrations of ara-A were >2-fold higher in blood of liver than in systemic circulation. Liver blood was collected from the inferior vena cava after closing below and above the outflows of the hepatic veins. The present result suggests that conjugation with galactosyl-terminating peptides might be a way to selectively increase the concentrations of NAs not only in hepatocytes, which have the asialoglycoprotein receptor, but also in cells infiltrating the liver, such as neoplastic cells of micrometastases nourished by hepatic sinusoids. BIOCHEM PHARMACOL **59**;3: 301–304, 2000. © 1999 Elsevier Science Inc.

KEY WORDS. asialoglycoprotein receptor; liver targeting of drugs; lactosaminated poly-L-lysine; antiblastic nucleoside analogs; regional anticancer chemotherapy; hepatic micrometastases

A selective delivery of antiviral and antiblastic NAs§ to parenchymal liver cells can be obtained by conjugation with galactosyl-terminating macromolecules [1–4], which enter hepatocytes via the asialoglycoprotein receptor [5, 6]. Since NAs, when set free from the carrier within hepatocytes, partly exit from these cells into the bloodstream [1, 4, 7–9], we considered the possibility that administration of galactosyl-terminating conjugates of NAs could result in plasma concentrations of these drugs that would be higher in liver sinusoids than in capillaries of other organs. In order to verify the validity of this hypothesis, we injected rats, in the present experiments, with a conjugate of ara-A with L-poly(LYS) and measured the plasma concentrations of ara-A in the blood of liver, aorta, and inferior vena cava. L-Poly(LYS) is a galactosyl-terminating carrier which allows preparation of conjugates with a high drug load [10]. Ara-A is an anticancer and antiviral NA [11]. In laboratory animals (mice, woodchucks), L-poly(LYS)–ara-A conjugate was selectively taken up by liver cells, where ara-A was released in an active form [12]. In subchronic and chronic (26 weeks) toxicity studies in rats and monkeys, this conjugate displayed a good tolerability.‖ Among synthesized L-poly(LYS) conjugates of NAs (i.e. with ara-A [10], iododeoxyuridine [13], ribavirin [8], fluorodeoxyuridine [14], or difluorodeoxycytidine [4]), ara-A was chosen for this study since it can be easily measured in plasma after administration of pharmacologically active doses [15].

MATERIALS AND METHODS

Conjugate

A poly(LYS) · HBr (Sigma Chemical Co.) with an average molecular mass, determined by viscosity, of 34.9 kDa was used. L-Poly(LYS)–ara-A and the two radioactive conjugate preparations were synthesized and characterized as described by Di Stefano *et al.* [10]. [D-glucose-1-^{14}C]lactose and [2,8 ^{3}H]adenine arabinoside were obtained from Amersham International and Moravek, respectively.

‡ Corresponding author: Prof. L. Fiume, Dipartimento di Patologia Sperimentale, via San Giacomo 14, 40126 Bologna, Italy. Tel. +39-0512094721; FAX +39-0512094746; E-mail: lfiume@alma.unibo.it

§ *Abbreviations:* ara-A, adenine arabinoside; ara-AMP, ara-A monophosphate; L-poly(LYS), lactosaminated poly-L-lysine; L-poly(LYS)–ara-A, conjugate of L-poly(LYS) with ara-A; and NA, nucleoside analog.

Received 2 April 1999; accepted 2 August 1999.

‖ Oberto G, Vigna E, Peano S, Ammannati E, Bussi R, Piccioli B and Peretti G, Istituto di Ricerche Biomediche Antoine Marxer, RBM SpA, Colleretto Giacosa, Italy; Exp. nes 970152 and 971057. Data on file at Laboratori Baldacci, Pisa, Italy.

TABLE 1. Chemical characteristics of L-poly(LYS)–ara-A preparations

Conjugate	Lactose (μg) / Conjugate (mg)	ara-A (μg) / Conjugate (mg)	% ε-NH_2 substituted by: Lactose	% ε-NH_2 substituted by: Ara-A	Sp act (dpm/μg)
L-Poly(LYS)–ara-A	202	356	29	65	
[^{14}C]L-Poly(LYS)–ara-A	224	321	30	55	1640
L-Poly(LYS)–ara-[^{3}H]A	270	281	35	47	2460

Animals

Male Wistar rats weighing 180–200 g were used. They were obtained from Harlan Italy and were maintained in an animal facility at the Department of Experimental Pathology, Bologna, receiving humane care in accordance with the guidelines of the Italian Ministry of Health. Rats were fed a standard pellet diet *ad lib.*

Determination of Ara-A in Blood of Liver and of Systemic Circulation

Rats received ara-A or L-poly(LYS)–ara-A via the dorsal vein of the penis under isoflurane anesthesia. The animals were anesthesized a second time at different intervals. A transverse laparotomy was performed and the xiphoid process was held up by two Mosquito clips. The falciform right and left triangular ligaments of the liver were dissected. The small intestine was folded on the left side of the rat to expose the retroperitoneum. The infrahepatic inferior vena cava above the right renal vein was encircled with a 3.0 silk suture. The aorta below the left renal vessels was cleaned of the surrounding connective tissue, and about 1 mL of blood was withdrawn from the distal aspect of the vessel; a microclamp was placed to occlude the aortic orifice in order to avoid any bleeding. After ligation of the infrahepatic vena cava, approximately 1 mL of blood was withdrawn from the vessel; again, a microclamp was placed to occlude the vena cava orifice to avoid hemorrhage. Finally, ca. 1 mL of blood was withdrawn from the suprahepatic inferior vena cava that had been cross-clamped above the outflows of the hepatic veins with a Satinsky clamp, grasping a small margin of the diaphragm. Rats were killed while under anesthesia. The blood samples were collected using heparinized syringes into tubes containing heparin (200 USP [16] units) and 20 μL of a solution (0.5 mg/mL in saline) of 2′-deoxycoformicin, an inhibitor of ara-A deamination [11]. Tubes were immediately centrifuged at 4° to remove blood cells. After addition of 37.5 μg of thymidine as internal standard to monitor the recovery, 300–500 μL of plasma was precipitated with 1 mL of

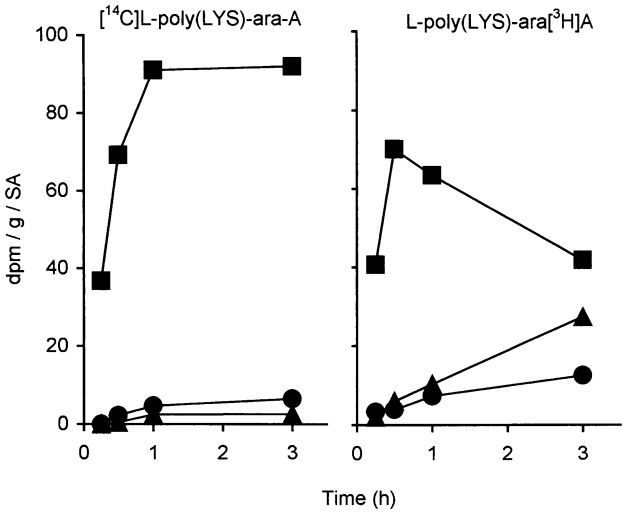

FIG. 1. Distribution of radioactivity (dpm/g/specific activity [SA]) in liver (■), spleen (▲), and intestine (●) of rats injected with [^{14}C]L-poly(LYS)–ara-A and L-poly(LYS)–ara[^{3}H]A. The conjugates were administered via the dorsal vein of the penis under isoflurane anesthesia at the dose of 6 μg/g. Radioactivity was measured as described by Di Stefano *et al.* [4]. Each entry represents the mean value of results from two to three animals. SE ranged from 1 to 3% of mean values.

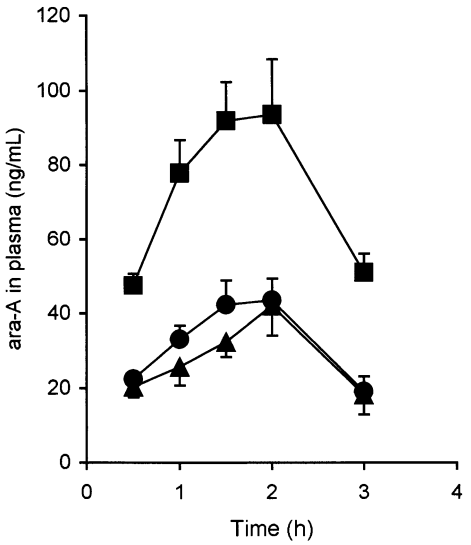

FIG. 2. Concentrations of ara-A in blood of liver (■), aorta (●), and inferior vena cava (▲) of rats i.v. injected with L-poly(LYS)–ara-A (5.6 μg/g, equal to 2 μg/g of ara-A). For each time, 5–9 animals were used. Data are mean values ± SE. Statistical analysis was performed by repeated measures ANOVA using Bonferroni's correction for multiple comparisons. At all times, the difference between the blood levels in liver and those in aorta and vena cava were found to be statistically significant, with *P* values ranging from <0.01 to <0.001. Trapezoidal areas under the curve (0–3 hr) of ara-A plasma levels in blood of liver, aorta, and cava were 204.5, 91.1, and 82.4 ng · hr/mL, respectively.

TABLE 2. Plasma concentrations (μg/mL) of L-poly(LYS)–ara-A in blood

Time (hr)	Hepatic veins*	Aorta	Vena cava
1	25.6 ± 3.4	30.7 ± 3.4	25.4 ± 0.2
2	16.4 ± 0.4	16.5 ± 1.0	16.1 ± 0.3

Data are the mean values ± SE from three rats.
*Blood of hepatic veins was collected from suprahepatic inferior vena cava, as described in Materials and Methods.

ethanol containing 10% ethyl acetate. After centrifugation, the supernatant was dried under vacuum and re-dissolved with 1 mL of water. Ara-A was measured by the HPLC method of McCann *et al.* [15].

Determination of L-Poly(LYS)–ara-A

The conjugate was determined by splitting the phosphoamide bond which links the 5′-phosphorylated derivative of ara-A (ara-AMP) to L-poly(LYS) [10] and measuring the released ara-AMP by the method of McCann *et al.* [15]. The procedure described by Fiume *et al.* [17] was followed, with the difference that incubation in 6% trichloroacetic acid at 50° was prolonged for 2 hr.

RESULTS AND DISCUSSION

In L-poly(LYS)–ara-A, the drug, phosphorylated in its primary OH group, is linked to the ε-amino groups of L-poly(LYS) by a phosphoamide bond [10]. The chemical characteristics of the conjugate preparations used in the present experiments are reported in Table 1. When L-poly(LYS)–ara-A was incubated in rat blood for 2 hr at 37°, neither ara-A nor ara-AMP was set free from the carrier (data not shown). Figure 1 shows the levels of radioactivity in liver, spleen, and intestine of rats i.v. injected with the conjugates labeled in the lactose or the ara-A moiety. The ratios between the amounts of radioactivity in liver and those in spleen and intestine were higher in animals injected with the former conjugate. This finding is consistent with a selective entry of the conjugate into liver cells and a release of the drug and/or its metabolites from these cells into the bloodstream with re-distribution to the tissues. In rats i.v. injected with ara-A (2 μg/g) or L-poly(LYS)–ara-A (5.6 μg/g, equal to 2 μg/g of ara-A) plasma concentrations of ara-A were measured in blood of aorta, inferior vena cava, and liver. Liver blood was collected from the inferior vena cava after closing below and above the outflows of the hepatic veins (see Materials and Methods). In agreement with data in humans [18], ara-A disappeared rapidly from plasma of rats injected with unconjugated drug and could not be detected at 30 min. Seven minutes after administration, the plasma concentrations of ara-A (ng/mL) in blood of aorta, vena cava, and liver were 54.5 ± 5.5, 54.2 ± 2.9, and 53.6 ± 6.8, respectively (mean values ± standard error from 4 rats). In animals injected with the conjugate, ara-A could be measured in plasma for up to 3 hr. Confirming the hypothesis which prompted this study, the area under curve (1–3 hr) of the plasma concentrations of the drug was >2-fold higher in blood of liver than in blood of aorta and vena cava (Fig. 2). One and two hours after injection of L-poly(LYS)–ara-A, the plasma levels of the conjugate were also measured. The concentrations were found to be very similar in the three blood samples (Table 2).

To our knowledge, this is the first reported observation of a prodrug which, after peripheral venous injection, produced higher drug plasma levels in liver than in systemic circulation. Conjugates of L-poly(LYS) with antiblastic NAs, such as that with fluorodeoxyuridine [14], can be exploited to improve the chemotherapy of tumors giving metastases in liver. Doses of conjugates producing drug levels in systemic circulation similar to those obtained after administration of unconjugated NAs should result in higher drug concentrations in liver blood. Without decreasing the therapeutic power of the NAs in organs apart from liver, the conjugates should enhance their activity against micrometastases supplied by liver sinusoid blood. In the adjuvant chemotherapy of colorectal cancer, concentrations of fluorouracil that are higher in liver sinusoids than in systemic circulation are obtained by infusing the drug into the portal vein via a catheter inserted during the surgical resection of the primary tumor [19–21]. Peripheral venous administration of galactosyl-terminating conjugates might have advantages over the intraportal infusion of drugs by assuring a better patient compliance, avoiding the risk of catheter complications and, most importantly, by permitting repeated cycles of treatment.

This work was supported by AIRC, MURST, the University of Bologna (funds for selected research topics), and Laboratori Baldacci, Pisa. The authors thank Dr. Christine M. Betts for correcting the English text.

References

1. 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.
2. Torrani Cerenzia M, 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 human albumin, administered for 4 weeks to patients with chronic type B hepatitis, decreased viremia without producing significant side effects. *Hepatology* **23:** 657–661, 1996.
3. Fiume L, Di Stefano G, Busi C, Mattioli A, Bonino F, Torrani

Cerenzia M, Verme G, Rapicetta M, Bertini M and Gervasi GB, Liver targeting of antiviral nucleoside analogues through the asialoglycoprotein receptor. *J Viral Hepat* **4:** 363–370, 1997.
4. Di Stefano G, Busi C, Mattioli A, Derenzini M, Trere D and Fiume L, Inhibition of [^{3}H]thymidine incorporation into DNA of rat regenerating liver by 2′,2′-difluorodeoxycytidine coupled to lactosaminated poly-L-lysine. *Biochem Pharmacol* **57:** 793–799, 1999.
5. Morell AG, Irvine RA, Sternlieb I, Scheinberg IH and Ashwell G, Physical and chemical studies on ceruloplasmin. V. Metabolic studies on sialic acid-free ceruloplasmin *in vivo*. *J Biol Chem* **243:** 155–159, 1968.
6. Hyodo I, Mizuno M, Yamada G and Tsuji T, Distribution of asialoglycoprotein receptor in human hepatocellular carcinoma. *Liver* **13:** 80–85, 1993.
7. 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 rat. *J Hepatol* **20:** 681–682, 1994.
8. Di Stefano G, Colonna FP, Bongini A, Busi C, Mattioli A and Fiume L, Ribavirin conjugated with lactosaminated poly-L-lysine: Selective delivery to the liver and increased antiviral activity in mice with viral hepatitis. *Biochem Pharmacol* **54:** 357–363, 1997.
9. Fiume L, Di Stefano G, Busi C, Incitti F, Rapicetta M, Farina C, Gervasi GB and Bonino F, Coupling to lactosaminated poly-L-lysine reduces the toxic effects of ribavirin on red blood cells. *J Hepatol* **29:** 1032–1033, 1998.
10. 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.
11. Cass CE, 9-β-D-Arabinofuranosyladenine (AraA). *Antibiotics* **5:** 85–109, 1979.
12. 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-lisine and administered by intramuscular route. *Hepatology* **22:** 1072–1077, 1995.
13. Biessen EA, Beuting DM, Vietsch H, Bijsterbosch MK and Van Berkel TJ, Specific targeting of the antiviral drug 5-iodo 2′-deoxyuridine to the parenchymal liver cells using lactosylated poly-L-lysine. *J Hepatol* **21:** 806–815, 1994.
14. Di Stefano G, Busi C, Derenzini M, Trere D and Fiume L, Conjugation of 5-fluoro-2′-deoxyuridine with lactosaminated poly-L-lysine to reduce extrahepatic toxicity in the treatment of hepatocarcinomas. *Ital J Gastroenterol Hepatol* **30:** 173–177, 1998.
15. McCann WP, Hall LM, Siler W, Barton N and Whitley RJ, High-pressure liquid chromatographic methods for determining arabinosyladenine-5′-monophosphate, arabinosyladenine, and arabinosylhypoxanthine in plasma and urine. *Antimicrob Agents Chemother* **28:** 265–273, 1985.
16. Majerus PW, Broze GJ, Miletich JP and Tollefsen DM, Anticoagulant, thrombolytic and antiplatelet drugs. In: *Goodman and Gilman's The Pharmacological Basis of Therapeutics* (Eds. Hardman JG, Limbird LE, Molinoff PB, Ruddon RW and Goodman Gilman A), pp. 1341–1359. McGraw-Hill, New York, 1996.
17. Fiume L, Mattioli A, Busi C, Corzani S and Di Stefano G, High pressure liquid chromatographic method for determining adenine arabinoside monophosphate coupled to lactosaminated albumin in plasma. *Pharm Acta Helv* **66:** 230–232, 1991.
18. Preiksaitis JK, Lank B, Ng PK, Brox L, LePage GA and Tyrrell DL, Effect of liver disease on pharmacokinetic and toxicity of 9-β-D-arabinofuranosyladenine-5-phosphate. *J Infect Dis* **144:** 358–364, 1981.
19. Taylor I, Machin D, Mullee M, Trotter G, Cooke T and West C, A randomized controlled trial of adjuvant portal vein cytotoxic perfusion in colorectal cancer. *Br J Surg* **72:** 359–363, 1985.
20. Liver Infusion Meta-analysis Group, Portal vein chemotherapy for colorectal cancer: A meta-analysis of 4000 patients in 10 studies. *J Natl Cancer Inst* **89:** 497–505, 1997.
21. Rougier P, Sahmoud T, Nitti D, Curran D, Doci R, De Waele B, Nakajima T, Rauschecker H, Labianca R, Pector JC, Marsoni S, Apolone G, Lasser P, Couvrer ML and Wils J, Adjuvant portal-vein infusion of fluorouracil and heparin in colorectal cancer: A randomised trial. *Lancet* **351:** 1677–1681, 1998.