Biochemical Pharmacology, Vol. 57, pp. 793–799, 1999.

ISSN 0006-2952/99/$–see front matter
PII S0006-2952(98)00359-1

Inhibition of [^{3}H]Thymidine Incorporation into DNA of Rat Regenerating Liver by 2′,2′-difluorodeoxycytidine Coupled to Lactosaminated Poly-L-Lysine

*Giuseppina Di Stefano, Corrado Busi, Alessandro Mattioli, Massimo Derenzini, Davide Trerè and Luigi Fiume**

Department of Experimental Pathology, University of Bologna, Bologna, Italy

ABSTRACT. The expression of asialoglycoprotein receptor (ASGP-R) on human hepatocarcinoma cells might be exploited to reduce the extrahepatic toxicity of DNA synthesis inhibitors by their conjugation with galactosyl- terminating peptides. We conjugated 2′,2′-difluorodeoxycytidine (dFdC), an inhibitor of DNA synthesis active on solid tumors, with lactosaminated poly-L-lysine (L-poly(LYS)). In experiments *in vitro*, L-poly(LYS)-dFdC inhibited proliferation of Hep G2 cells, a human hepatocarcinoma cell line which maintains the ASGP-R. Inhibition was rescued by asialofetuin. To study the pharmacological action of the conjugate *in vivo*, we used rats 18–24 hr after 2/3 hepatectomy and observed that regenerating hepatocytes expressed ASGP-R on their surface and internalized L-poly(LYS)-dFdC. Conjugate uptake by bone marrow, spleen, and intestine was negligible. We also found that L-poly(LYS)-dFdC inhibited [^{3}H]thymidine incorporation into DNA of regenerating liver. These results indicated that hepatectomized rats were a suitable animal model to study the pharmacological action, on DNA-synthesizing hepatocytes, of conjugates binding to ASGP-R and carrying inhibitors of DNA synthesis. L-poly(LYS)-dFdC also inhibited [^{3}H]thymidine incorporation in bone marrow, spleen, and intestine. Evidence was obtained that inhibition of DNA synthesis in extrahepatic tissues was a consequence of drug release from hepatocytes into blood-stream after the bond with the carrier has been broken down within liver cells. Possible ways of reducing the exit of dFdC from liver cells, thereby obtaining an inhibition of DNA synthesis restricted to dividing hepatocytes, were discussed. BIOCHEM PHARMACOL **57**;7: 793–799, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. drug targeting; asialoglycoprotein receptor; hepatocellular carcinoma; anticancer chemotherapy; 2′,2′-difluorodeoxycytidine; lactosaminated poly-L-lysine

The ASGP-R† is a glycoprotein present in large amounts only on hepatocytes, where it is expressed mainly on the sinusoidal surface. It mediates uptake and lysosomal degradation of circulating galactosyl-terminating glycoproteins [1]. The presence of the ASGP-R on hepatocytes has been exploited to reduce the extrahepatic side effects of antiviral nucleoside analogs (NAs) active against hepatitis viruses [2, 3]. NAs are conjugated with peptides exposing galactosyl residues; the conjugates are selectively taken up by hepatocytes, where lysosomal enzymes split the bond between the carrier and the drug, allowing the latter to become concentrated in liver cells.

A similar strategy was suggested [4–6] to increase the chemotherapeutic index of antiblastic drugs in the treatment of those hepatocarcinomas which maintain the ASGP-R [7] (80% of the well-differentiated forms‡). Inhibitors of DNA synthesis coupled to galactosyl-terminating carriers should kill the neoplastic proliferating hepatocytes without damaging either normal hepatic parenchyma, in which the large majority of cells do not divide [8], or proliferating cells of extrahepatic tissues, which do not have the receptor [1].

In the present experiments, we coupled dFdC (gemcitabine) to L-poly(LYS). dFdC is a nucleoside analog which inhibits DNA synthesis and is active in the treatment of solid tumors [9]; L-poly(LYS) is a hepatotropic carrier which allows preparation of conjugates with a very heavy drug load [10]. This property is important because it permits the intracellular penetration of the required amount of drug even when the number of ASGP-R molecules on hepato-

* Corresponding author: Prof. L. Fiume, Dipartimento di Patologia Sperimentale, Via San Giacomo 14, 40126 Bologna, Italy. Tel. +39-051-354721; FAX +39-051-354746.

† *Abbreviations:* ara-AMP, adenine arabinoside monophosphate; ASGP-R, asialoglycoprotein receptor, BrdU, 5-bromo-2′-deoxyuridine; dFdC, 2′,2′-difluorodeoxycytidine; dFdCMP, dFdC 5′-monophosphate; dFdCMPlm, imidazolide of dFdCMP; L-poly(LYS), lactosaminated poly-L-lysine; L-poly(LYS)-dFdC, conjugate of L-poly(LYS) with dFdC; NT, nucleoside transporter; and PH, partial (2/3) hepatectomy.

Received 25 June 1998; accepted 8 October 1998.

‡ Trerè D, Fiume L, Badiali De Giorgi L, Di Stefano G, Migaldi M and Derenzini M, manuscript submitted for publication.

carcinoma cells is reduced. The inhibitory effect of L-poly(LYS)-dFdC on DNA synthesis of dividing hepatocytes was studied in experiments *in vitro* and *in vivo*. *In vitro*, the action of conjugate was investigated on Hep G2 cells, a human hepatocarcinoma cell line which maintains the ASGP-R [11]. In the experiments *in vivo*, the conjugate was administered to rats 18–24 hr after PH, when hepatocytes synthesize DNA [12]. We preliminarily verified that regenerating hepatocytes maintained the ASGP-R on their surface at this time point and that the bond between dFdC and L-poly(LYS) was stable in blood of hepatectomized rats.

MATERIALS AND METHODS

Preparation of Conjugates

Poly(LYS)hydrobromide with a molecular mass ranging from 28,500 to 38,800 Da was obtained from Sigma Chemical Co. Coupling of lactose was performed as described in [13]. L-poly(LYS) was dialyzed (first against NaCl 0.9% and finally against water) and lyophilized. Lactose content was measured by the phenol–sulphuric acid method of Dubois *et al.* [14]. Conjugation of dFdC (Eli Lilly) was obtained via the imidazolide of dFdCMP [15]. The drug was phosphorylated in its primary OH group according to Yoshikawa *et al.* [16]. dFdCMP was purified by chromatography on a Dowex-1-formate column eluted with a formic acid gradient (0–4 M) and was recovered by lyophilization. HPLC using an anionic exchange column showed that the preparations were at least 95% pure. After dephosphorylation with *Escherichia coli* alkaline phosphatase type III (Sigma), the dFdCMP preparations, analyzed by HPLC using a μ-Bondapak C_{18} column, displayed only one peak which had the same retention time as an authentic sample of the corresponding nucleoside. dFdCMP was converted to its imidazolide (dFdCMPlm) according to the procedure of Lohrman and Orgel [17]. The HPLC using a μ-Bondapak C_{18} column showed that the imidazolide preparations were at least 85% pure. dFdC coupling via dFdCMPlm was obtained as described for conjugation of ara-AMP to L-poly(LYS) [10]. Briefly, L-poly(LYS) (100 mg) was dissolved in 2 mL 0.5 M sodium carbonate buffer, pH 11; 400 mg of dFdCMPlm was added and the pH re-adjusted to 11 with 5 M NaOH. After incubation at 50° for 96 hr, the conjugate was diafiltered with 0.9% NaCl. Chemical characterization of the conjugate was carried out by assaying the coupled drug spectrophotometrically and measuring lactose as described above. We found that the sugar moiety of dFdC did not interfere with lactose determination. The poly(LYS) content of conjugate was calculated from the amount of lactose, the lactose/poly(LYS) weight ratio having been determined before drug coupling. This was possible because the bond between the sugar and the lysine ε-NH_2 groups does not break down during conjugation [10].

A radioactive conjugate was obtained by labeling L-poly(LYS) with [^{3}H]sucrose, which was coupled using [^{3}H]raffinose (5.1 Ci/mmol) (NEN Life Science) according to the procedure of Van Zyle *et al.* [18].

Experiments on Hep G2 Cells

Hep G2 cells were obtained from Prof. F. Dall'Olio, Department of Experimental Pathology, University of Bologna. They were routinely tested for mycoplasma contamination by Hoechst 33258 (Sigma) and found to be negative. Expression of ASGP-R was verified by measuring the uptake of asialofetuin labeled with [^{3}H]formaldehyde (NEN Life Science). The effect of free and coupled dFdC on cell proliferation was studied as described in [6]. ID_{50} (doses producing a 50% inhibition of cell growth) of free and coupled drug were calculated from the polynomial regression of the experimental data plotted as log dose versus % inhibition. r^2 values were 1 and 0.99 for free and coupled dFdC, respectively.

Animals

Male Wistar rats weighing 180–200 g were used. They were obtained from Harlan Nossan 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*. pre- and postoperatively and were maintained on 12-hr light–dark cycles. They were subjected to PH in the morning under ether anesthesia, according to Higgins and Anderson [19].

ASGP-R Immunolabeling

Samples of regenerating liver taken from 5 rats 24 hr after PH were fixed in 10% neutral-buffered formalin and routinely paraffin-embedded. From each paraffin block, 3–4 μm thick sections were cut, cleared in xylene, and rehydrated. Slides were then removed from water to plastic slide holders, fully immersed in 10 mM sodium citrate buffer (pH 6.0), and heated for 20 min at 120° in a commercially available pressure cooker. After cooling to room temperature in the sodium citrate buffer, slides were treated with a solution of 0.3% H_2O_2 in methanol for 30 min at room temperature to abolish endogenous peroxidase activity. Sections were then incubated for 10 min in a moist chamber with non-immune rabbit serum diluted to 5% in PBS pH 7.2–7.4 to reduce non-specific background staining. Sections were then incubated at overnight at 4° with the primary antibody (anti-ASGP-R monoclonal antibody, purchased from Daiichi Pure Chemical Co. Ltd.) diluted 1:200. This antibody was produced by Kohgo *et al.* [20] by immunizing Balb-C mice with the ASGP-R purified from human liver. It was found to also bind to the rat receptor [20]. Sections were subsequently incubated with rabbit anti-mouse immunoglobulin biotinylated antibody (DAKO) diluted 1:600 in PBS with 5% normal human AB serum for 30 min, and then in a streptavidin-biotin-

peroxidase preformed complex (DAKO) for 30 min. The immunologic reaction was developed using 3,3′-diamino-benzidine-tetra-hydro-chloride-H_2O_2 solution, dehydrated, and mounted with Eukitt (O. Kindler, Germany). A negative control was performed by omitting the anti-ASGP-R monoclonal antibody.

BrdU Immunolabeling

Twenty-two hours after PH, five rats received an intraperitoneal injection of BrdU (Sigma) (100 μg/g). After 2 hr, the rats were killed and liver specimens fixed in 10% neutral-buffered formalin and routinely paraffin-embedded.

Sections were de-waxed and treated with a solution of methanol/H_2O_2 0.3% as previously described. They were then treated with 4N HCl at room temperature for 20 min to improve DNA staining and washed with double-distilled water and PBS pH 7.2–7.4 for 10 min. The sections were incubated in a moist chamber with non-immune rabbit serum diluted 1:50 in PBS for 10 min and left to incubate for 1 hr at room temperature with the primary antibody (anti-BrdU monoclonal antibody, Beckton Dickinson) diluted 1:500. The immunoreaction was developed as previously described for ASGP-R. A negative control was performed by omitting the specific anti-BrdU monoclonal antibody.

The BrdU-positive nuclei were counted directly at the microscope in 10 consecutive fields at 25X magnification, and were related to the number of total nuclei counted in 10 consecutive fields at the same magnification in a serial histological section stained with hematoxilin and eosin.

Stability of the Bond between dFdC and L-poly(LYS) in Blood

Twenty-four hours after hepatectomy, rats were bled from the retro-orbital plexus under ether anesthesia. Heparinized blood was incubated at 37° with or without L-poly(LYS)-dFdC (250 μg/mL). At 0 time and after 4 hr, blood cells were removed by centrifugation and 1 mL plasma was exhaustively washed with NaCl 0.9% using a 3000 Da cut-off YM3 membrane (Amicon Corp.), which retains the conjugate. The samples were then incubated at 50° in the presence of 5% trichloroacetic acid (TCA) for 2 hr. This procedure completely split the phosphoamide bond between dFdCMP and L-poly(LYS), leaving the dFdCMP molecule unchanged. The precipitated material was removed by centrifugation and TCA was extracted with water-saturated ethyl ether. Before incubation with TCA, uridine (40 μg/mL) was added as internal standard to monitor the recovery. dFdCMP was quantified by HPLC on an 8PSAX column (Waters, Millipore Corp.) calibrated with an authentic sample of this compound. The conditions of chromatography were as in [21]. The interference of the extracts from the blood samples not containing the conjugate was calculated and subtracted. It only represented 1.7–2.2% of the area of the dFdCMP peak.

Organ Distribution of the Conjugate

Two normal and two hepatectomized rats were used. [^{3}H]L-poly(LYS)-dFdC was injected into the back muscle of hind leg at the dose of 5 μg/g, in a total volume of 100 μL NaCl 0.9%. In hepatectomized rats, administration was performed 18 hr after PH. Six hours after injection, the animals were killed under ether anesthesia, and the liver, spleen, and a tract of intestine (8 cm long starting from pylorus) were rapidly removed and homogenized in 4 vol. (wt/vol) of water. Bone marrow was ejected from the two femurs into a preweighed scintillation vial by blowing air with a syringe. It was suspended with 1 mL of water and dissolved with soluene. Radioactivity was measured as described in [21].

[^{3}H]Thymidine Incorporation into DNA

[^{3}H]Thymidine (Amersham International) (sp. act. 25 Ci/mmol) was intraperitoneally injected (0.05 μCi/g body weight) 23.5 hr after PH. Free and coupled dFdC (or saline, in control animals) were injected 0.5–6 hr before [^{3}H]thymidine into the back muscle of hind leg in a total volume of 100 μL NaCl 0.9%. Thirty minutes after [^{3}H]thymidine administration, animals were killed under ether anesthesia, and liver, a tract of intestine (8 cm long starting from pylorus), spleen, and bone marrow from femurs were rapidly removed. DNA was extracted by the method of Schneider and Greco [22], and the radioactivity was determined and the concentration measured according to Burton [23].

Biological Assay of dFdC in Plasma

Twenty-three hours after PH, rats were i.m. injected with saline or with 1 μg/g of coupled dFdC and were bled 1 hr later from retro-orbital plexus under ether anesthesia. Two animals were used for each group. In order to remove the high molecular weight components, including the conjugate, 1 mL of plasma was diluted with water to 10 mL and diafiltered using a 3000 Da cut-off membrane until 1 mL of the initial volume remained. The diafiltrate was lyophilized, dissolved with 1 mL of PBS and, after sterilization through a 0.22 μm filter, added (25–100 μL) to Hep G2 cell cultures. The cultures with added plasma diafiltrate from rats injected with saline were used as controls. Inhibition of cell proliferation caused by plasma diafiltrate from animals treated with the conjugate was measured as described above and its rescue by deoxycytidine, an antagonist of dFdC [24], was assessed.

RESULTS

Chemical Characteristics of Conjugate

One milligram of conjugate contained 351 μg of poly(LYS), 302 μg of lactose, and 347 μg of dFdCMP, corresponding to 262 μg of dFdC. Considering that 1 mg of poly(LYS) contains 7.8 μmol of lysine residues and that the

TABLE 1. Effect of free and conjugated dFdC on Hep G2 cell proliferation

Exp	Compounds	Concentration (ng/mL)	Inhibition (%) of cell growth
1	dFdC	5	100*
	dFdC	2.5	100
	dFdC	1.25	75 (S)
	dFdC	0.6	5 (NS)
2	L-poly(LYS)-dFdC	37 (10)†	100
	L-poly(LYS)-dFdC	18.5 (5)	100
	L-poly(LYS)-dFdC	9.3 (2.5)	62 (S)
	L-poly(LYS)-dFdC	4.6 (1.25)	12 (NS)
3	L-poly(LYS)-dFdC	18.5 (5)	100
	Asialofetuin	10^6	18 (NS)
	L-poly(LYS)-dFdC +Asialofetuin	18.5 (5) 10^6	29 (NS)

Experiments were performed as described in [6]. Cells (7×10^5) were seeded; after 24 hr, the compounds were added and cells from two wells were harvested and counted. Each dose of compound was tested in duplicate. After a further 24 hr cells from treated cultures and from two untreated wells were counted. The number of cells formed during the period in which the compounds were present was calculated. Each experiment was carried out twice. Results were statistically evaluated by means of Student's *t*-test, and the difference was considered significant (S) or not significant (NS) for $P < or > 0.05$, respectively.

*100% inhibition means that in these cultures, 24 hr after the addition of the compounds, the number of cells was unchanged or even decreased.

†The amounts of dFdC contained in the conjugate are shown in parentheses.

molecular weights of lactose and dFdC are 342 and 247.2, respectively, it can be calculated that the ϵ-NH_2 groups of poly(LYS) were substituted by the sugar and by the drug at the percentage of 32 and 39, respectively. One-milligram of [^{3}H]L-poly(LYS)-dFdC contained 2 μg of [^{3}H]sucrose, 297 μg of dFdC, and 275 μg of lactose. Specific activity was 1.6×10^6 dpm/mg.

Effect of Conjugate on Hep G2 Cells

The effect of the conjugate on the proliferation of dividing hepatocytes maintaining the ASGP-R was studied *in vitro* using Hep G2 cells [11]. Results are shown in Table 1. The ID_{50}s, calculated as described in Materials and Methods, were 1.11 and 2.13 ng/mL for the free and the conjugated drug, respectively. Inhibition by L-poly(LYS)-dFdC was rescued by asialofetuin, indicating that the conjugate entered Hep G2 cells through the ASGP-R.

Incorporation of BrdU and Expression of ASGP-R in Regenerating Liver

Twenty-four hours after PH, immunocytochemical detection of incorporated BrdU revealed that a great number of hepatocyte nuclei (29.1%) were labeled (Fig. 1). After immunolabeling for ASGP-R, all hepatocytes exhibited a clear plasma membrane staining (Fig. 2). This result was in agreement with the finding that most (about 70%) of the rat hepatocyte ability to bind asialo-orosomucoid was maintained 24 hr after PH [25].

Stability of the Bond between dFdC and L-poly(LYS) in Blood

In spite of the release of liver lysosomal enzymes in blood-stream after PH [26], the bond between dFdC and L-poly(LYS) was stable in blood of hepatectomized rats. When the conjugate was incubated at 37° for 4 hr in blood

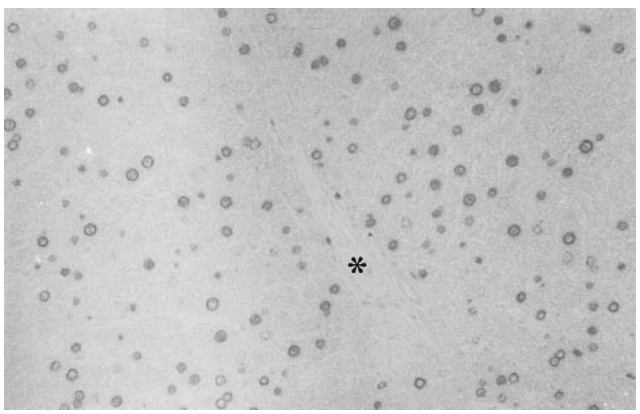

FIG. 1. BrdU immunostaining of a section of regenerating liver 24 hr after PH. The periportal zone is shown. A large percentage of hepatocyte nuclei is stained (X 200). The asterisk indicates a portal vein branch.

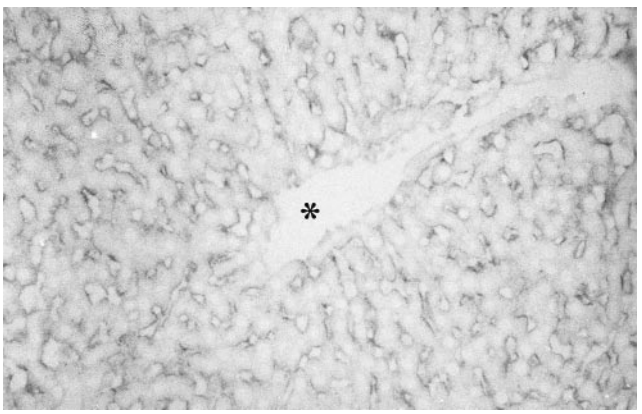

FIG. 2. ASGP-R immunostaining of a section of regenerating liver 24 hr after PH. The periportal zone is shown. The sinusoidal surface of hepatocytes is stained (X 200). The asterisk indicates a portal vein branch.

TABLE 2. Organ distribution of radioactivity after administration of [^{3}H]L-poly(LYS)-dFdC to normal and hepatectomized rats

	dpm/g/sp. act.			
	Liver	Bone marrow	Intestine	Spleen
Normal rats	39.69 ± 6.64	1.18 ± 0.80	0	0.07 ± 0.07
Hepatectomized rats	57.87 ± 2.34	0.54 ± 0.32	0	1.54 ± 1.27

[^{3}H]L-poly(LYS)-dFdC (specific activity = 1600 dpm/μg) was injected i.m. at the dose of 5 μg/g. In hepatectomized rats, administration was performed 18 hr after hepatectomy. Rats were killed 6 hr after injection. Radioactivity in the organs was measured as described in Materials and Methods.

withdrawn from rats 24 hr after PH, only 1.7% of the drug was released.

Organ Distribution of the Conjugate

Organ distribution was studied by using a conjugate labeled with [^{3}H]sucrose. Since this disaccharide is not hydrolyzed in mammalian lysosomes and does not readily diffuse from these organelles [27], the radioactivity of [^{3}H]sucrose-labeled peptides remains within the entered cells and consequently allows the organ uptake of peptides to be measured precisely [18]. As shown in Table 2, the conjugate was selectively taken up by the liver of both normal and hepatectomized animals. Since the weight of liver was 8.1 ± 0.2 and 3.7 ± 0.2 in normal and hepatectomized rats, respectively, it can be calculated that conjugate uptake by the whole organ was 321.5 and 214.4 μg, respectively. The amounts of conjugate internalized by bone marrow, spleen, and intestine were negligible.

Effect of the Conjugate on [^{3}H]thymidine Incorporation into DNA

Table 3 shows the effect of free and coupled dFdC on [^{3}H]thymidine incorporation in liver, bone marrow, spleen, and intestine of hepatectomized rats. In previous experiments, it was observed that 48 hr after PH, the ability of regenerating rat hepatocytes to internalize and digest galactosyl-terminating peptides was completely lost [25]. The finding that L-poly(LYS)-dFdC administered 18–20 hr after PH, inhibited [^{3}H]thymidine incorporation into liver indicates that regenerating rat hepatocytes not only internalized the conjugate at this time point (Table 2), but could also transport it to lysosomes, where the bond between the drug and the carrier was split.

L-poly(LYS)-dFdC also inhibited [^{3}H]thymidine incorporation in bone marrow, spleen, and intestine. Moreover, the ratios between the percentages of inhibition in liver and other organs were not higher in rats treated with the conjugate than in rats injected with the free drug, indicating that in the former animals liver targeting of the inhibitory action of dFdC did not occur (see Discussion).

Biological Assay of dFdC in Plasma

Since the bond between dFdC and L-poly(LYS) was stable in blood of hepatectomized rats and the conjugate did not penetrate into cells of bone marrow, spleen, and intestine (Table 2), inhibition of DNA synthesis in these organs was probably due to partial release of dFdC from hepatocytes in blood-stream when the drug was set free from the carrier within these cells. To verify this hypothesis, we searched for free dFdC in plasma of hepatectomized rats after i.m.

TABLE 3. Effect of free and coupled dFdC on [^{3}H]thymidine incorporation into DNA in organs of hepatectomized rats

			% Inhibition			
Compound	Dose (μg/g)	Time* (hr)	Liver	Bone marrow	Spleen	Intestine
Free dFdC	0.3	1	0	50.1 (S)	ND†	0
	0.5	0.5	0	34.4 (S)	26.2 (NS)	15.6 (NS)
	0.5	1	38.3 (S)	56.2 (S)	ND	0
	0.5	2	42.3 (NS)	57.5 (S)	59.4 (NS)	24.5 (NS)
	1	1	71.0 (S)	57.4 (S)	86.2 (S)	17.4 (NS)
Coupled dFdC	0.3	1	0	0	18.2 (NS)	11.3 (NS)
	0.5	2	0	0	25.3 (NS)	12.3 (NS)
	1	2	12.9 (NS)	51.1 (S)	34.4 (NS)	1.1 (NS)
	1	4	63.5 (S)	78.3 (S)	84.1 (S)	9.9 (NS)
	1	6	74.4 (S)	87.8 (S)	93.3 (S)	56.8 (S)

Rats were killed 24 hr after hepatectomy. [^{3}H]Thymidine (sp. act. 25 Ci/mmol) (0.05 μCi/g body weight) was intraperitoneally administered 0.5 hr before killing. Compounds were i.m. injected 0.5–6 hr before [^{3}H]thymidine. For each dose and time interval, three animals were treated and three hepatectomized rats, i.m. injected with saline, served as controls. Results (% inhibition of [^{3}H]thymidine incorporation in treated versus control animals) were evaluated by means of Student's *t*-test. The difference was considered significant (S) or not significant (NS) for $P < $ or > 0.05, respectively.

*Time before [^{3}H]thymidine injection.

†ND = not determined.

administration of the conjugate (3.7 μg/g, corresponding to 1 μg/g dFdC). Since plasma concentrations of dFdC were too low to be detected by HPLC, we studied the inhibitory effect of plasma diafiltrate on Hep G2 cell proliferation (see Materials and Methods).

Fifty μL of the plasma diafiltrate from hepatectomized animals i.m. administered with the conjugate produced a statistically significant inhibition (90%) of cell proliferation. Deoxycytidine (0.5 mM) completely removed this inhibition, indicating that it was caused by dFdC [24].

DISCUSSION

In the present experiments, L-poly(LYS)-dFdC, administered to rats 18–20 hr after PH, was selectively taken up by regenerating liver, where it inhibited [^{3}H]thymidine incorporation into DNA. This result indicates that, at this time point after PH, rats can be used as an animal model for preliminary studies of conjugates binding to ASGP-R and carrying inhibitors of DNA synthesis. Conjugates which inhibit DNA synthesis in regenerating hepatocytes can be selected for evaluation of their anticancer activity in long-term experiments using rats with chemically induced hepatocarcinomas maintaining the receptor [28].

In spite of the selective uptake by the liver, L-poly(LYS)-dFdC conjugate also inhibited DNA synthesis in bone marrow, spleen, and intestine. Inhibition of DNA synthesis in these organs was probably due to partial release of dFdC from hepatocytes in blood-stream when the drug was set free from the carrier within these cells. This was supported by the finding that plasma diafiltrate from hepatectomized rats treated with the conjugate inhibited Hep G2 cell growth, and that this inhibition was rescued by deoxycytidine. A similar drug release from liver was observed in animals administered with galactosyl-terminating conjugates of ara-AMP [29] and ribavirin [13, 21], two antiviral nucleoside analogs. In the case of these drugs, the advantage of conjugation was maintained in spite of the partial exit of the drugs from the liver. In patients with chronic hepatitis B, the chemotherapeutic index of ara-AMP was increased by conjugation [30], and in monkeys the anemia produced by ribavirin was significantly lower when the drug was administered in the coupled form [31]. On the contrary, in the present experiments L-poly(LYS) coupling did not accomplish a liver targeting of the inhibitory action of dFdC. A larger release from liver of dFdC compared to ara-AMP and ribavirin might explain the incapacity of L-poly(LYS)-dFdC to restrict the pharmacological action of the drug to hepatocytes.

Permeation of nucleosides across the cell membrane is in large part mediated by membrane proteins (NTs) which transport nucleosides and their analogs into and out of the cell [32]. In the case of dFdC, membrane permeation also occurs via passive diffusion [9]. Two ways may be attempted to obtain an inhibition of DNA synthesis restricted to dividing hepatocytes: (a) conjugation of inhibitors of DNA synthesis with a lower NT affinity than dFdC and unable to cross the cell membrane by passive diffusion. These drugs, coupled to L-poly(LYS), should be forced to enter hepatocytes by receptor-mediated endocytosis and when released free from the carrier should be transported from the liver cells to blood-stream and from blood-stream to other cells in smaller amounts; and (b) the same result might be obtained by administering along with the dFdC conjugate an inhibitor of NTs such as dipyridamole, which was found to counteract the action of dFdC in various cancer cell lines [33] and has been used in clinical studies to hinder the transmembrane transport of nucleosides [34].

The authors thank Dr. Christine Betts for revising the manuscript. This work was supported by grants from AIRC, MURST, and the University of Bologna (funds for selected research topics).

References

1. Geffen I and Spiess M, Asialoglycoprotein receptor. *Int Rev Cytol* **137:** 181–219, 1992.
2. Fiume L, Mattioli A, Balboni PG, Tognon M, Barbanti-Brodano G, De Vries J and Wieland TH, Enhanced inhibition of virus DNA synthesis in hepatocytes by trifluorothymidine coupled to asialofetuin. *FEBS Lett* **103:** 47–51, 1979.
3. Fiume L, Di Stefano G, Busi C, Mattioli A, Bonino F, Torrani Cerenzia M, Verme G, Rapicetta M, Bertini M and Gervasi G, Liver targeting of antiviral nucleoside analogues through the asialoglycoprotein receptor. *J Viral Hep* **4:** 363–370, 1997.
4. Schneider YJ, Abarca J, Aboud-Pirak E, Baurain R, Ceulemans F, Deprez-De Campeneere D, Lesur B, Masquelier M, Otte-Slachmuylder C, Rolin-van Swieten D and Trouet A, Drug targeting in human cancer chemotherapy. In: *Receptor-mediated Targeting of Drugs*. NATO ASI Series A: Life Sciences (Eds. Gregoriadis G, Poste G, Senior J and Trouet A), vol. 82, pp. 1–25. Plenum Press, New York, 1984.
5. O'Hare KB, Hume IC, Scarlett L, Chytrý V, Kopeckovà P, Kopecek J and Duncan R, Effect of galactose on interaction of N-(2-hydroxypropyl)methacrylamide copolymers with hepatoma cells in culture: preliminary application to an anticancer agent, daunomycin. *Hepatology* **10:** 207–14, 1989.
6. Di Stefano G, Busi C, Derenzini M, Trerè 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.
7. Hyodo I, Mizuno M, Yamada G and Tsuji T, Distribution of asialoglycoprotein receptor in human hepatocellular carcinoma. *Liver* **13:** 80–85, 1993.
8. Arber N, Zajicek G and Ariel I, The streaming liver II. Hepatocyte life history. *Liver* **8:** 80–87, 1988.
9. Plunkett W, Huang P, Searcy CE and Gandhi V, Gemcitabine: preclinical pharmacology and mechanism of action. *Semin Oncol* **23:** 3–15, 1996.
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. Schwartz BA, Fridovich SE, Knowles BB and Lodish IIF, Characterization of the asialoglycoprotein receptor in a continuous hepatoma line. *J Biol Chem* **256:** 8878–8881, 1981.
12. Bucher NLR, Regeneration of mammalian liver. *Int Rev Cytol* **15:** 245–300, 1963.

13. 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.
14. 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.
15. 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.
16. Yoshikawa M, Kato T and Takenishi T, A novel method for phosphorylation of nucleosides to 5′-nucleotides. *Tetrahedron Lett* **50:** 5065–5068, 1967.
17. Lohrmann R and Orgel LE, Preferential formation of (2′-5′)-linked internucleotide bond in non-enzymatic reactions. *Tetrahedron* **34:** 853–855, 1978.
18. Van Zile J, Henderson LA, Baynes JW and Thorpe SR, [^{3}H]Raffinose, a novel radioactive label for determining organ sites of catabolism of protein in circulation. *J Biol Chem* **254:** 3547–3553, 1979.
19. Higgins GM and Anderson RM, Experimental pathology of the liver: I. Restoration of the liver of the white rat following partial surgical removal. *Arch Pathol* **12:** 186–202, 1931.
20. Kohgo Y, Kato J, Nakaya R, Mogi Y, Yago H, Sakai Y, Matsushita H and Niitsu Y. Production and characterization of specific asialoglycoprotein receptor antibodies. *Hybridoma* **12:** 591–598, 1993.
21. Di Stefano G, Bignamini A, Busi C, Colonna FP and Fiume L, Enhanced accumulation of ribavirin and its metabolites in liver versus erythrocytes in mice administered with the liver targeted drug. *Ital J Gastroenterol Hepatol* **29:** 420–426, 1997.
22. Schneider WC and Greco AE, Incorporation of pyrimidine deoxyribonucleosides into liver lipids and other components. *Biochim Biophys Acta* **228:** 610–626, 1971.
23. Burton K, A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem J* **62:** 315–323, 1956.
24. Graziadei I, Kelly T, Schirmer M, Geisen FH, Vogel W and Konwalinka G, Antitumor effect of the nucleoside analogs 2-chlorodeoxyadenosine and 2′,2′-difluorodeoxycytidine on human hepatoma Hep G2 cells. *J Hepatol* **28:** 504–509, 1998.
25. Howard DJ, Stockert RJ and Morell AG, Asialoglycoprotein receptors in hepatic regeneration. *J Biol Chem* **257:** 2856–2858, 1982.
26. Kanashima R, Nagasue N, Kobayashi M and Inokuchi K, A comparative study of lysosomal enzyme contents in blood and liver of rats following partial hepatectomy. *Hepato-Gastroenterol* **27:** 448–456, 1980.
27. Cohn ZA and Ehrenreich BA, The uptake, storage and intracellular hydrolysis of carbohydrates by macrophages. *J Exp Med* **129:** 201–225, 1969.
28. Harris L, Preat V and Farber E, Patterns of ligand binding to normal, regenerating, preneoplastic, and neoplastic rat hepatocytes. *Cancer Res* **47:** 3954–3958, 1987.
29. 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.
30. 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.
31. 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.
32. Cass CE, Nucleoside transport. In: *Drug Transport in Antimicrobial and Anticancer Chemotherapy* (Ed. Georgopapadakou NH), pp 403–451. Marcel Dekker, New York, 1995.
33. Jansen WJM, Pinedo HM, van der Wilt CL, Feller N, Bamberger U and Boven E, The influence of BIBW22BS, a dipyridamole derivative, on the antiproliferative effects of 5-fluorouracil, methotrexate and gemcitabine *in vitro* and in human tumor xenografts. *Eur J Cancer* **31A:** 2313–2319, 1995.
34. Wilson JKV, Fischer PH, Remick SC, Tutsch KD, Grem JL, Nieting L, Alberti D, Bruggink J and Trump DL, Methotrexate and dipyridamole combination chemotherapy based upon inhibition of nucleoside salvage in humans. *Cancer Res* **49:** 1866–1870, 1989.