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아시알로페투인을 약물수송체로 이용한 아시클로버의 간표적화

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Hepatic Targeting of Acyclovir Using Asialofetuin as a Drug Carrier

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With the purpose of improving the therapeutic index of [^{3}H] acyclovir (ACV) in the treatment of chronic hepatitis B infection, asialofetuin (AF) which after selective interaction with Ashwell's receptor specifically enters into hepatocytes, was chosen as a carrier system for hepatic targeting. This drug was first converted to its monophosphate (ACVMP), which was subsequently activated by water soluble carbodiimide to conjugate with ε-NH_2 groups of lysine residues of AF. The molar ratio of ACVMP to AF in the conjugate was 3.9. In rats, elimination of ACVMP-AF conjugate after i.v. injection showed two phase elimination kinetics. Initial apparent elimination rate constant in rats was 0.191 min^{-1} which was greater than that of ACV. The elimination rate constant from terminal phase was 0.021 min^{-1}. Area under the total radioactivities versus time curve was found to be several times larger in liver than in other organs (spleen, intestine, lung and kidney) after i.v. administration of the conjugate labelled in the drug moiety. The above results suggested that ACVMP-AF conjugate was rapidly taken up by hepatocytes and could be a useful hepatic targeting system.

Keywords—Hepatic targeting, Asialofetuin, Acyclovir, Ashwell receptor, Endocytosis

Introduction

The side or toxic effects of a drug result from the unwanted action of a drug on the non-targeted sites. A variety of methods are under development to overcome the undesired distribution of a drug to the non-targeted sites, or to increase selective transfer of a drug using specific carriers.[1] To accomplish this purpose, a subdivided receptor which exist exclusively on certain sites or lesions should be found and employed as a selective biological receptor for target molecules.[2] For the effective targeting of a drug, the carrier has to possess the target specificity. In this point of view, antibodies, ever since discovered, have been studied thoroughly as a candidate for drug carriers.[3] For example, anticancer agents or toxins which have a high cytotoxicity, after coupling to antibodies, can be linked to the surface of the specific cells. The conjugate bound to the surface of the cancer cell, can destroy the tumor cells after internalization by the endocytosis.[4] Besides antibodies, macromolecular drug carrier systems such as albumin, globulins and synthetic polymers

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have been developed in an attempt to enhance the selective action of cytotoxic agents in the field of cancer chemotherapy.[5,6] Synthetic soluble polymers have been much widely used as drug ca rriers than microbody forms did, since they have many targeting sites in the body.[3,7] Kramer *et al*[8] reported that the drug covalently linked to the bile acid could be specifically targeted to the liver. It was possible to target the anticancer agent, acrylophenone, to the specific cells by the use of low-density lipoprotein,[9] and galactosylated poly (L-lysine) as a hepatotropic vector of 9-β-D-arabino- furanosyladenine 5'-monophosphate and ACV (Fig. 1) accomplished a selective drug delivery to the hepatic cells in rats.[10] It is well recognized that receptor-mediated endocytosis is deeply involved in the biological effects of polypeptide, hormones, growth factors and other molecules.[11,12] The mammalian hepatic asialoglycoprotein receptor has shown to actively and efficiently degrade the various glycoconjugate containing terminal galactosyl groups or N-acetylgalactosamine residue[13] and desialylated serum glycoproteins in lysosome after internalization by the specific endocytosis.[14,15] The protein-receptor is transported into the hepatocytes, and hepatic receptor is not destroyed after internalization, and unoccupied receptor molecules are promptly returned to the cell surface while the asialoglycoprotein is digested in lysosomes.[16] Consequently, the rate of the removal of glycoproteins in the liver remains constant.[17] Based on these findings, AF was chosen as a carrier to selectively deliver ACV to hepatocytes on which ACV acts.

Hepatitis B can be spread by transfusion or many other pathways, and it is related to chronic hepatitis and primary hepatic cancer.[18] ACV alone or in combination with interferon can be used in the treatment of chronic type B hepatitis[19] preventing from the DNA replication of viruses. It, however, is necessary to increase the chemotherapeutic index of ACV due to the side effects of

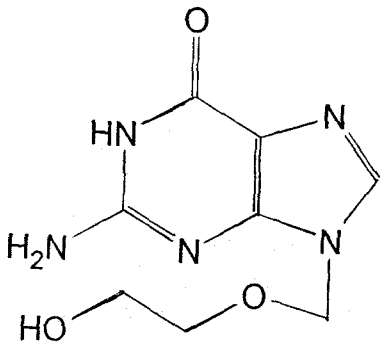

Figure 1—The structure of acyclovir.

ACV, such as decrease in spermatogenesis, nau sea, vomitting, mutation etc. The antiviral activity of ACV is dependent on phosphorylation of the compound, and triphosphated form is the most active. The thymidine kinase of uninfected cell by viruses cannot use ACV as a substrate effectively. The thymidine kinase encoded mainly by herpes virus can efficiently convert ACV to acyclovir monophosphate (ACVMP).[20] This ACVMP is then converted to a diphosphate by guanylate kinase and is subsequently converted to triphosphate by a cellular enzyme.[21] This acyclovir triphosphate (ACVTP) inhibits the DNA replication of viruses. ACV is mainly penetrated into herpes virus infected cells and specifically converted to active ACVTP. In the absence of herpes virus kinase, however, phosphorylation of ACV occurs to a limited extent in mammalian cells and consequently the activity of the drug against other viruses is relatively low.[22] In the experiments reported here, ACV was chemically phosphorylated to ACVMP, which was subsquently conjugated with AF, a glycoprotein which specifically penetrates liver cells. The conjugate released ACVMP in hepatocytes. The limiting step of enzymatic conversion of ACV to its monophosphate may be by-passed in liver by the conjugate.

Experimental

Materials

Asialofetuin, 1-ethyl-3-(3-dimethyl aminopropyl)-carbodiimide hydrochloride (EDC), dialysis tubing, trinitrobenzene- sulfonic acid (TNBS), sodium dodecyl-sulfate (SDS), SDS molecular weight markers 200 kit and Sephadex G-100 were purchased from Sigma Chemical Co. (St Louis, MO, USA), Soluene-350 (0.5 N quaternary ammonium hydroxide in toluene) was purchased from Packard Instrument Co. (Warrenville, IL, USA). ACV was kindly supplied by Kyung Dong Pharmaceuticals (Seoul, Korea) and [^{3}H] ACV was purchased from NEN (Wilmington, DE, USA). All other reagents were of reagent grade and obtained commercially.

Animals

Male Sprague-Dawley rats (Life Science Co., Taegu, Korea) weighing 200~250 g were used in this study. The animals were fasted for 24 hours before experiments in order to reduce the variation of hepatic metabolism and allowed to free access of water.

Synthesis of [^{3}H] acyclovir monophosphate

[^{3}H] ACVMP was synthesized according to the method of Yoshikawa[23] with slight modifications. 0.48 g of cold ACV in 5 m*l* of ethanol : water (3:7) were mixed with [^{3}H] ACV solution (250 μCi), and the mixture was evaporated to dryness *in vaccuo*. The dry solid was cooled to 0℃, and an ice-cold solution of $POCl_3$ (0.5 m*l*) in triethyl phosphate (5.4 m*l*) was added slowly with stirring. The flask was sealed with a drying tube and stirred with a magenetic bar for 2 hr at 0℃. Additional POCl3 (0.15 m*l*) was added slowly during the next 2 hr. The resulting clear mixture was poured into ice water (60 m*l*) and kept at 0-4℃ for 16 hr. The solution was diluted with 20 ml water, neutralized to pH 5.0 with concentrated NH4OH, and applied to the Dowex-1-formate column (1×30 cm). The column was washed with deionized double-distilled water until unreacted ACV free and eluted with a formic acid gradient (0-4 M). Fractions corresponding to ACVMP were pooled and reduced to dryness.

Synthesis of conjugate ([^{3}H] ACVMP-AF)

180 mg EDC dissolved in 600 μ*l* H_2O was added to 180 mg ACVMP dissolved in 300 μ*l* H_2O by addition of $NaHCO_3$ (pH 7.5). After 10 min at 25℃, 90 mg AF dissolved in 900 μ*l* H_2O (adjusted to pH 7 with NaOH) was added with stirring. The mixture was incubated at 25℃ for 24 hr, then chromatographed on a Sephadex G-100 column. The ratio of ACVMP to AF in the conjugates was calculated from the radioactivity measured by a liquid scintillation counter for dertermining ACVMP and the absorbance at 540 nm for measuring AF concentration by the method of Bradford[24] with a standard of immunoglobulin G. The ACVMP content of the conjugate was calculated by dividing the content of ACVMP by that of the protein.

Size exclusion chromatography

The conjugation of ACVMP to carrier AF was confirmed by size exclusion chromatography employing a Sephadex G-100 column (2.5×50 cm). The column was eluted with 0.15 M Sodium chloride solution, and the flow rate was 2 m*l*/min. The sample was dissolved in mobile phase, then 2 m*l* of the solution was loaded onto the column. The fractions corresponding to conjugate were collected, pooled and lyophilized. The lyophilized materials were dialyzed against water and lyophilized again.

SDS-PAGE of conjugate

The electrophoresis was accomplished by the method of Sambrook.[25] AF, ovalbumin (45 KD), bovine plasma albumin (66 KD), phosphoylase b (97.4 KD), and β-galactosidase (116 KD) were employed as M_r markers. Gels were stained with Coomassie brilliant blue for 4 hr and destained with the solution composed of MeOH : CH_3COOH : H_2O (30:10:60).

Pharmacokinetics and distribution study

The rats were maintained under ether anesthesia during the operation. The cannulas (PE 50,

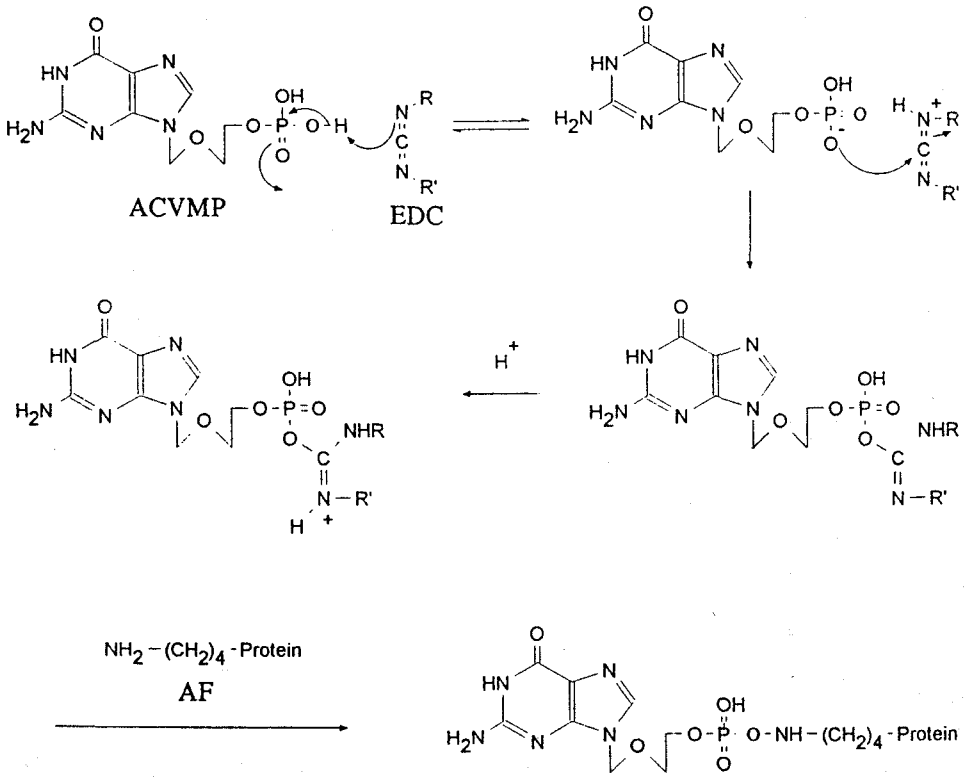

Figure 2—The synthetic pathway of ACVMP-AF conjugate.

Thomas scientific Co., NJ, USA) were placed surgically in the femoral vein and artery. A 10-min recuperative period was allowed before the study was begun. The 0.127 μCi (dissolved in 1 m*l* of injectable 0.9 % NaCl solution) of [^{3}H] ACV, and conjugate were injected via femoral vein of rat, repectively. Blood samples were collected with the appropriate time intervals. After each blood sampling, rats were sacrificed by cervical dislocation, and the lungs, liver, spleen, intestine and kidneys were removed, rinsed with cold normal saline, blotted dry, and weighed.

Analysis of total radioactivity

A portion of plasma and each organ were solubilized with 4 m*l* of Soluene 350 (0.5 N quaternary ammonium hydroxide in toluene, Packard Instrument Co.) per 1 g of organ in a counting vial. The vial was kept at 50℃ for 12 hr, and 0.2 m*l* of isopropyl alcohol and 0.4 m*l* of 30% hydrogen peroxide were added to minimize color quenching. In order to neutralize the solution, about 0.4 m*l* of 5 N HC*l* was added and then 10 m*l* of a scintillation cocktail. The total radioactivity in the biological sample was determined by the liquid scintillation counter (Packard, USA) after equilibration in the dark at 25℃ for at least 24 hr prior to counting.

RESULTS

Synthesis of [^{3}H] acyclovir monophosphate

The dry material obtained from the above method gave 1 spot on TLC developed with CH_3CN: 0.1 M NH_4Cl (7:3). Rf's for ACV and ACVMP were 0.62 and 0.17, respectively. The compound was further confirmed by ^{1}H NMR spectroscopy. The multiplicity of the protons attached to carbon next to phosphate group was hexet (triplet of doublet

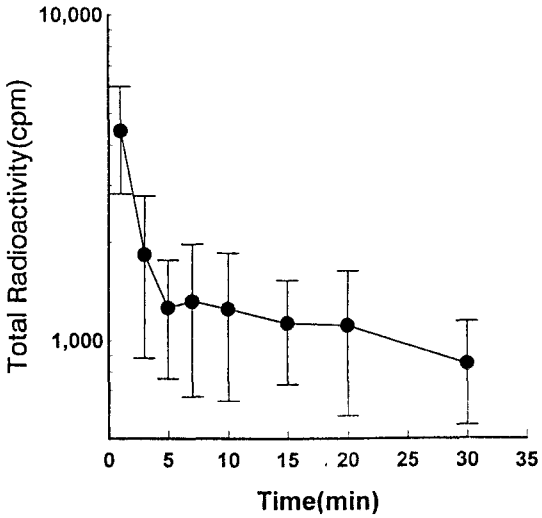

Figure 3—The pharmacokinetic profile of ACVMP-AF in rats after i.v. administration of [^{3}H] ACVMP-AF (0.27 μg/g, specific activity of 3.5×10^4 cpm/mg). Each point represents the mean value (± S.D.) of results from 3 animals.

between proton and phosphorus atom), which indicated that the compound possesed a phosphate group. The coupling constant between proton and phosphorus was 9.0 Hz.

Synthesis of conjugate ([^{3}H] acyclovir monophosphate-asialofetuin)

Conjugate ([^{3}H] ACVMP-AF) was prepared by a carbodiimide reaction according to the method of Fiume *et al.*[26] Carbodiimide activates the phosphoryl groups of the drug and reacts with nucleophiles such as amino groups of protein and forms the bond between the drug and protein (Fig. 2). Conjugates prepared at acidic medium became insoluble in the lyophilized state because of polymerization, the molar ratio drug/protein decreased after lyophilization.[26] Solubility of conjugates, prepared at pH 7.5 and higher pH 8.5 or 9.5, did not decrease on standing. As indicated by SDS gel electrophoresis (data not shown), the conjugate was composed primarily of a monomer as well as polymers of AF which were formed as a side reaction of carbodiimide coupling.

Measurement of ACVMP content of the con jugate

Table I—*Elimination Rate Constants of [^{3}H] ACV and [^{3}H] ACVMP-AF*

	ACV	ACVMP-AF
Λ_1	0.073 ± 0.028 (min^{-1})	0.191 ± 0.073 (min^{-1})
Λ_2	0.009 ± 0.004 (min^{-1})	0.021 ± 0.010 (min^{-1})

Each entry represents the mean value (± S.D.) of results from 3 animals.

[^{3}H] ACV and [^{3}H] ACVMP-AF were administered intravenously in rats at a dose of 0.27 μg/g (specific activity, 3.5×10^4 cpm/mg) and 2 μg/g (specific activity, 13.6×10^6 cpm/mg), respectively.

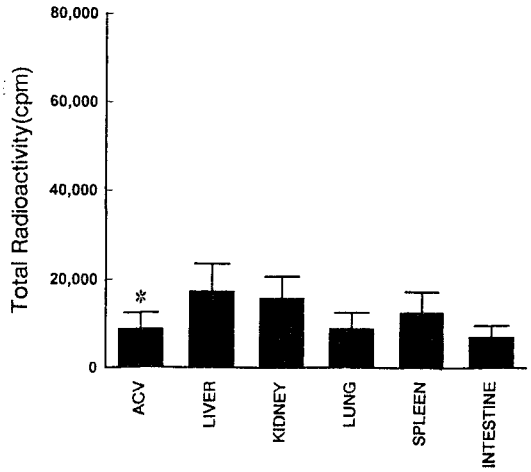

Figure 4—The organ distribution of ACVMP-AF, 5 min. after i.v. administration of [^{3}H] ACVMP-AF at a dose of 0.27 μg/g (specific activity, 3.5×10^4 cpm/mg). *ACV represents total radioactivity in liver obtained by i.v. administration of [^{3}H] ACV at a dose of 2 μg/g, (specific activity, 13.6×10^6 cpm/mg). Each entry represents the mean value (± S.D.) of results from 3 animals.

The ACVMP contents of the conjugates were determined as molar ratio by measuring the concentration of the protein and radioactivity of conjugate. The molar ratio of ACVMP/AF was 3.9.

Pharmacokinetics and organ distribution

As shown in Fig. 3, elimination of [^{3}H] ACVMP-AF conjugate after i.v. injection showed two phase elimination kinetics. Initial apparent elimination rate constant in rats was 0.191 min^{-1} which was greater than ACV (0.073 min^{-1}) (Table 1). The elimination rate constant from terminal phase was

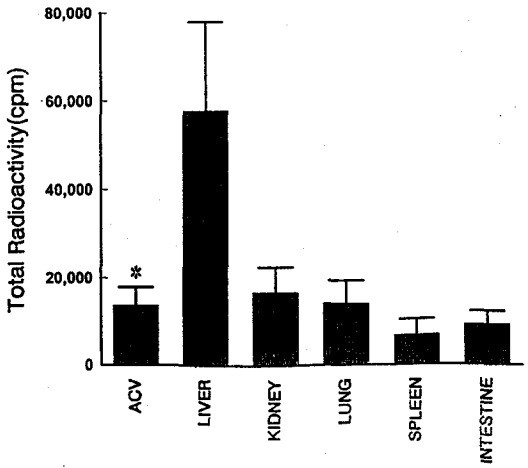

Figure 5—The organ distribution of ACVMP-AF, 12 min. after i.v. administration of [^{3}H] ACVMP-AF at a dose of 0.27 μg/g (specific activity, 3.5×10^4 cpm/mg). *ACV represents total radioactivity in liver obtained by i.v. administration of [^{3}H] ACV at a dose of 2 μg/g, (specific activity, 13.6×10^6 cpm/mg). Each entry represents the mean value (± S.D.) of results from 3 animals.

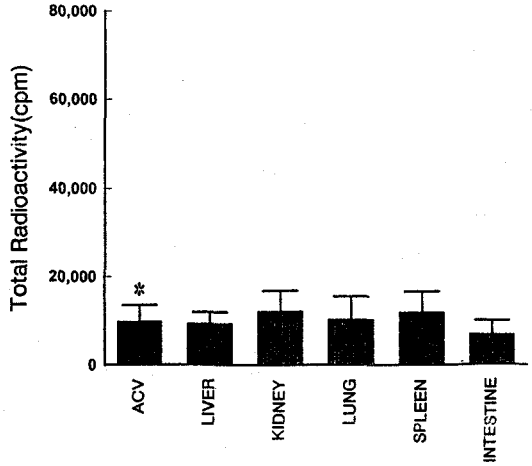

Figure 6—The organ distribution of ACVMP-AF, 30 min. after i.v. administration of [^{3}H] ACVMP-AF at a dose of 0.27 μg/g (specific activity, 3.5×10^4 pm/mg). *ACV represents total radioactivity in liver obtained by i.v. administration of [^{3}H] ACV at a dose of 2 μg/g, (specific activity, 13.6×10^6 cpm/mg). Each entry represents the mean value (± S.D.) of results from 3 animals.

0.021 min^{-1}. Free [^{3}H] ACV was injected i.v. at a dose of 2 μg/g and [^{3}H] ACVMP-AF at doses of 0.27 μg/g which corresponded to 40 μg conjugate respectively. Free and coupled [^{3}H] ACVMP had the specific activity of 13.6×10^6 cpm/mg and 3.5×10^4 cpm/mg. In rats injected with [^{3}H] ACV, radioactivity was equally distributed in liver, spleen and intestine; higher values were found in kidneys.[33] Table 2 showed that area under the total radioactivities versus time curve was found to be several times larger in liver than in other organs (spleen, intestine, lung and kidney) after i.v. administration of the conjugate labelled in the drug moiety. AUTR in liver from conjugate was also greater than that from [^{3}H] ACV. As shown in Fig. 4. 5. and 6, radioactivity of liver measured at 12 min after i.v. injection of [^{3}H] ACVMP-AF conjugate was much higher than those in the other organs, implicating a rapid elmination from circulation into the liver. Radioactivity of liver measured at 12 min after i.v. injection of [^{3}H] ACV was significantly lower than that of [^{3}H] ACVMP-AF, which might be explained in part by the fact that ACV is mainly eliminated via kidney intact.

DISCUSSION

The antiviral actions of ACV evidently involve its selective phosphorylation. Phosphorylation to monophosphate is efficiently performed by a virus-coded kinase which is present in herpes-virus- infected cells.[20] The monophosphate is further converted to ACV triphosphate by cellular enzymes.[22] In the absence of herpes virus kinase, phosphorylation of ACV occurs to a limited extent in mammalian cells and consequently the activity of the drug against other viruses is relatively low.[22] Based on the lysosomotropic approach to antiviral chemotherapy,[27] ACV was conjugated with AF, in order to introduce selectively in hepatocytes and to reduce its side effects in the treatment of chronic hepatitis B.

As implicated in Table 2, selective delivery of

Table II—*Area under the Total Radioactivities versus Time (AUTR) from Each Organ after i.v. Administration of [^3H] ACVMP-AF in Rats*

Liver	843,214 ± 198,235 (cpm×min)	Lung	293,738 ± 79,462 (cpm×min)
Kidney	401,745 ± 95,359 (cpm×min)	Intestine	188,471 ± 56,341 (cpm×min)
Spleen	246,813 ± 67,304 (cpm×min)	*Liver	297,247 ± 68,403 (cpm×min)

Each entry represents the mean value (± S.D.) of results from 3 animals.
[^{3}H] ACV and [^{3}H] ACVMP-AF were administered at a dose of 0.27 μg/g (specific activity, 3.5×10^4 cpm/mg). *AUTR obtained by i.v. injection of [^{3}H] ACV at a dose of 2 μg/g (specific activity, 13.6×10^6 cpm/mg). AUTR was estimated from time 0 to 30 min.

ACV to liver should result in a more efficient inhibition of virus replication accompanied by a lower toxicity for the other tissues. In recent studies, Hubbard and coworkers[28,29] have used electron microscopy to delineate the liver cell type target, kinetics of internalization, and intracellular fate of ^{125}I-labeled and also ferritin- and horse radish peroxidase-conjugated asialoglycoproteins. They showed that ^{125}I-labeled AF is bound rapidly and specifically by liver parenchymal cells and internalized in rats. These results are reflected in the rapid elimination of ACVMP-AF conjugate after i.v. administration in rats (Fig. 3). The new ACVMP-AF conjugate fulfils the criteria required for a lysosomotropic drug-carrier complex and posseses the properties to accomplish liver targeting of ACV. It maintains the capacity of AF to interact with the specific receptors on the surface of hepatocytes with which it can freely come in contact since hepatic sinusoids are not barrier for proteins. The conjugate is selectively taken up by the liver where the AF carrier is digested. The hepatic receptor for galactosyl terminating glycoproteins is not destroyed after internalization following ligand binding and unoccupied receptor molecules are promptly returned to the cell surface from an internal pool.[16] Consequently the hepatic uptake of galactosyl-terminating glycoproteins remains constant with time.[17] The released drug remains in high concentration in liver and its pharmacological action is confined to this organ. In this regard, ara-AMP conjugation with lactosaminated albumin was also investigated as a potential lysosomotropic drug-carrier complex.[30] The unique occurrence of asialoglycoprotein receptor in hepatocytes should be of great value in optimizing site-directed killing of target tissues in vivo by this conjugate. It is reported that asialofetuin linked with arabinoside A[31,32] and lactosaminated albumin conjugated with ACVMP released active moieties in hepatocytes.[33] The fragment A of diphtheria toxin was linked to asialofetuin, the conjugate was 1800 times as toxic as diphtheria toxin on rat hepatocytes.[34] An investigation focusing on the chemical linkage of ACV and the effects on the nature of the conjugate as a site-specific macromolecular prodrug is in progress. Further experiments are in progress to determine whether the conjugate release ACVMP in hepatocytes.

In conclusion, ACVMP-AF conjugate was successfully synthesized and purified in our laboratory. Distribution of the radiolabelled conjugates was liver concentrated. Results obtained from the pharmacokinetic and distribution study suggested that ACVMP-AF conjugate was rapidly taken up by hepatocytes and could be a useful hepatic targeting system.

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