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STUDIES ON THE UPTAKE OF LOW MOLECULAR WEIGHT MONOMERIC TRIS-GALACTOSYL CONJUGATES BY THE RAT LIVER

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Abstract—We have attempted to direct low molecular weight compounds to the liver via the internalizing asialoglycoprotein receptor on parenchymal cells by conjugation to a monomeric triantennary galactosyl cluster. Acetate and a hypolipidaemic ansamycin were derivatized and the biodistribution of the conjugates was determined 250 sec and 30 min after administration to Wistar rats. The ansamycin conjugate (CGH46) was rapidly cleared from the circulation by the liver; after 250 sec, 64% of the radiolabelled dose was found in the liver compared to 18% in the blood. However, the distribution of the conjugate did not differ significantly from that of unconjugated ansamycin (CGH45). Tris-galactosyl acetate showed no capacity to localize in the liver, with only 2% recovered from that organ 250 sec after administration compared to 38% in the blood and 13–18% in the kidneys, skin and muscle. Extraction efficiency of CGH46 by isolated perfused rat livers was almost 20% of the administered dose and this value was not significantly changed by co-administration of specific inhibitors of the uptake process. It is concluded that derivatization of low molecular weight molecules with monomeric triantennary galactosyl residues is unlikely to increase their affinity for the liver.

Drugs with a pharmacological action in the liver are likely to be rendered more effective by selective delivery to this organ. Hepatocytes express classes of receptors located on the plasma membrane that could, through drug/ligand–receptor interactions with subsequent endocytosis and intracellular drug release, be exploited for targeted drug delivery. The asialoglycoprotein (ASGP‡) receptor has attracted the attention of a large number of investigators attempting delivery of active molecules to the parenchymal cells of the liver [1, 2]; this constitutive [3] receptor is found exclusively on and in hepatocytes of mammalian liver [4, 5], binds and internalizes a number of galactose- or *N*-acetylgalactosamine-terminated ligands with high affinity and is expressed in relatively large numbers at the cell surface [6]. Its physiological role appears to be the clearance by binding, endocytosis and intracellular degradation of plasma-derived ASGP [5]. Following internalization, ligands and drug–asialoglycoprotein conjugates are routed to lysosomes where they are degraded and drug released, although a proportion appear to be returned to the cell surface and thus escape degradation [7].

In addition to its ability to bind a variety of naturally occurring and modified ASGP, the ASGP receptor recognises a number of synthetic low molecular weight ligands. For example, Connolly *et al.* [8] synthesized cluster glycosides containing either one, two or three galactose residues per ligand and established that the mono-, bis- and tris-galactosides showed increasing affinity for the rabbit hepatocyte receptor as determined by their ability to inhibit the binding of [^{125}I]asialoorosomucoid (ASOR). These and other observations by this group [9, 10] provide strong evidence that the ASGP receptor preferentially recognises three galactose or galactosamine [11] moieties arranged in a defined triangular configuration. Such ligands may have a higher affinity for the receptor and more binding sites than natural ligands. Similar triantennary mannosyl conjugates have been used to investigate the delivery of dexamethasone to macrophages [12].

As repeated administration of foreign glycoproteins as drug carriers is likely to be undesirable, we have studied the potential of low molecular weight tris-galactosyl drug conjugates for drug targeting to the liver. Two model compounds were conjugated to tris-galactosyl residues. One was a derivative of Rifamycin SV (CGH45) that had shown significant hypolipidaemic activity [13] and is representative of a class of large (M_r 1082) lipophilic drugs [14]. The other was the low molecular weight hydrophilic compound acetate. We have studied the specific and non-specific liver targeting properties of these tris-galactosyl conjugates by measuring liver

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‡ Abbreviations: ASGP, asialoglycoprotein; ASOR, asialoorosomucoid; ASF, asialofetuin; GalNAc, *N*-acetyl-D-galactosamine; tris-gal-acetate, *N*-[tris[(β-D-galactopyranosyloxy)methyl]methyl]-*N*α-(acetyl)glycinamide.

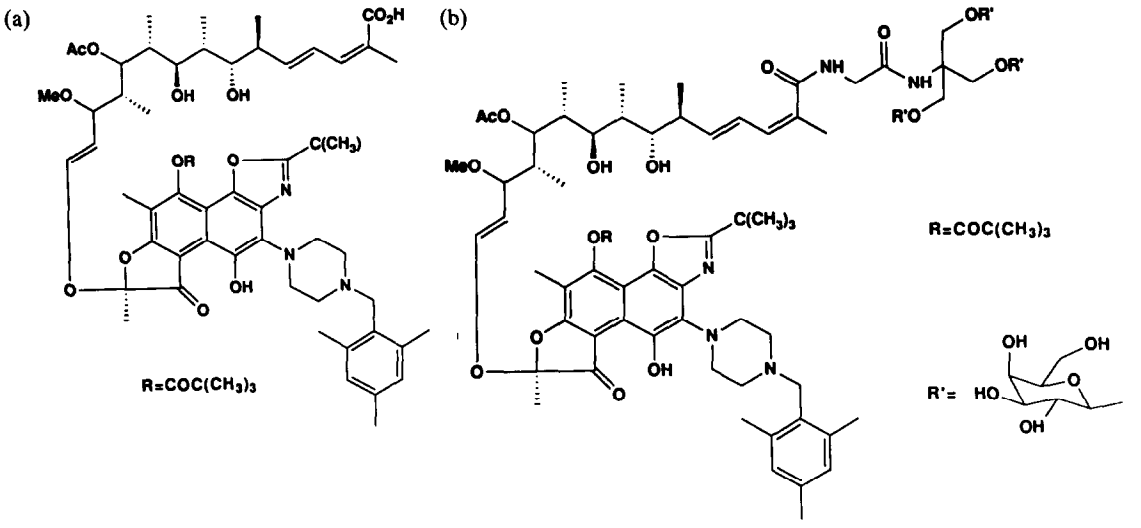

Fig. 1. Structures of CGH45 (a) and CGH46 (b).

uptake *in vivo* and in isolated perfused liver in the presence and absence of specific ligands competing for the ASGP receptor.

MATERIALS AND METHODS

Compounds. The open chain hypolipidaemic ansamycin 8-*O*-trimethylacetyl-3-[*N'*-(2,4,6-trimethylbenzyl)-*N*-piperazinyl]-25-(acetyloxy)-4,21,23-trihydroxy-27-methoxy-7,12,16,20,22,24,26-heptamethyl-(epoxypentadeca[1,11,13]-trienimino)furonaphtho[2,1-*d*]-(2'-tertiarybutyl)-oxazole-11-oxo-15-carboxylic acid (CGH45; Fig. 1a) was synthesized from Rifamycin SV by introduction of a piperazinyl residue in the C3 position followed by acylation and thermolysis as described by Menear *et al.* [13]. [^{14}C]CGH45 (sp. act. 1.853 MBq/mg) was also prepared as described previously [13]; the single ^{14}C atom was located in the piperazine moiety. In order to produce the tris-galactosyl conjugate CGH46 (Fig. 1b), CGH45 or [^{14}C]CGH45 was activated by reaction with a slight excess of disuccinimidyl carbonate; the TLC-purified product was then reacted with *N*-[tris[(β-D-galactopryanosyloxy)methyl]methyl]glycine hydrochloride, prepared essentially as described by Kempen *et al.* [15] in the presence of one equivalent of *N*-methyl morpholine. After TLC recovery, purity was greater than 95% and the specific activity of the radiolabelled conjugate was 1.025 MBq/mg. These synthetic procedures will be described in more detail elsewhere.

[^{3}H]*N*-[Tris[(β-D-galactopyranosyloxy)methyl]-methyl]-N^{α}-(acetyl)glycinamide (tris-gal-acetate) was prepared by adding a solution of [^{3}H]acetic acid sodium salt (37.0 MBq; New England Nuclear, Stevenage, U.K.) in 100 μL ethanol to 10 mg *N*-[tris[(β-D-galactopyranoxy)methyl]methyl]glycine hydrochloride salt in 0.5 cm^3 dimethylformamide containing 6 mg hydroxybenzatriazole and 4 mg 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide at room temperature under an atmosphere of dry nitrogen. After 3 hr, 1 μL glacial acetic acid was added and the reaction mixture stirred for a further 24 hr. Water (0.5 mL) was then added and the reaction product recovered by passing the mixture through an Animex A-14 (H-form) ion exchange column and an AG 50W-X4 cation exchange column, eluting with water pH 7. Removal of the solvent under reduced pressure afforded the reaction product as a clear colourless oil (6.2 mg, 58% recovery). The reaction product (M_r 696) was homogeneous as determined by TLC (1-butanol:acetic acid:pyridine:water 15:3:10:12) and ^{1}H NMR (90 MHz).

Orosomucoid was obtained from the Sigma Chemical Co. (Poole, U.K. and desialylated with neuraminidase to yield asialoorosomucoid as described by Grant and Kaderbhai [16]. ASOR was iodinated using the chloramine-T method [17], purified by Sephadex G-25 gel filtration chromatography and dialysed against three changes of 31 phosphate-buffered saline. Asialofetuin (ASF) and *N*-acetyl-D-galactosamine (GalNAc) were purchased from Sigma. CGH46 was dissolved in dimethyl sulphoxide prior to mixing with rat plasma for *in vivo* distribution studies of perfusion medium (bicarbonate Krebs–Ringer oxygenated with 95% O_2:5% CO_2, containing 10 mM L-lactic acid) for liver perfusion studies. All other compounds were dissolved in physiological saline or liver perfusion medium as appropriate.

Animals. Male Wistar rats (250–350 g; Bantam and Kingman, Hull, U.K.) were fed *ad lib.* both before and during the course of the studies; experiments were carried out under sodium pentobarbitone anaesthesia (60 mg/kg).

In vivo *distribution of radiolabelled compounds.* Compounds were injected in a volume of 400 μL into the tail vein; immediately prior to injection, solutions were centrifuged to remove any particulate material. Each dose contained approximately 100,000 dpm. Rats were killed by cervical dislocation either 250 sec or 30 min after injection. Major organs were weighed and samples of liver, lung, kidney, spleen, intestine (ileum), adrenals, testes, bladder and urine, skin, muscle, fat, heart, brain, thymus, eye, salivary gland, blood and injection site (tail segment) were obtained. All tissue and blood

samples were combusted to water and CO_2 using a 306 Tissue Oxidiser (Canberra Packard, Pangbourne, U.K.) and assayed for ^3H or ^{14}C activity in a LS1801 scintillation counter (Beckman High Wycombe, U.K.). Tissue samples from animals injected with [^{125}I]ASOR were counted using a 5650 auto-γ counter (Canberra Packard). Total body mass for blood, skin, muscle, fat and intestine was estimated as described by Crandall and Drabkin [18].

In some experiments, the rate of extraction of compounds from the circulation was determined using blood samples withdrawn every 15 sec from the cannulated carotid artery.

Isolated liver perfusion. The hepatic portal vein of Wistar rats was cannulated *in situ* and the liver (weight range 9.4–13.2 g) was excised and supported on a funnel. Livers were perfused in a non-recirculating mode through the portal vein at 38° with perfusion buffer employing a flow rate of 18.3 mL/min. After a 10 min equilibrium period, the compounds under study were perfused for 5 min; this was followed by a 10 min perfusion period with perfusion medium alone to enable the delayed release of compounds from the liver to be assessed. In competition studies, the competitor substance was introduced into the perfusion medium at the same time as the test compound. The perfusate distal to the liver was sampled at 0.5–1 min intervals and analysed by HPLC or scintillation counting. Glucose production from lactic acid was estimated by measuring glucose concentration in the perfusate with a Glucose GOD-Perid test kit (Boehringer Mannheim, Germany) to ensure continued metabolic activity of the liver [19].

HPLC determination of CGH45 and CGH46 in liver perfusate. Samples were mixed with 2.5 vol. of methanol and centrifuged at 1800 *g* for 5 min to remove proteins; 100 μL of the supernatant were then injected onto a Waters gradient HPLC system (Millipore U.K., Watford, U.K.) fitted with a 5 μm 3.9 × 150 mm C18 column (Altech, Carnforth, U.K.) maintained at 38°. The mobile phase was composed of solvent A (water adjusted to pH 2.2 with HCl) and solvent B (80% acetonitrile, 20% methanol), and a linear gradient applied with a solvent A: solvent B starting ratio of 40:60 and a final ratio at 17 min of 10:90. The flow rate was 1.15 mL/min and the column was allowed to equilibrate in the initial solvent ratio for 8 min. The elution profile was measured with a Waters 441 single wavelength detector at 254 nm.

RESULTS

Distribution of i.v. administered compounds in the Wistar rat

The rate of disappearance of CGH45 and CGH46 from the blood stream of male Wistar rats was determined over a 200 sec period. As can be seen from Fig. 2, the clearance of the conjugate (CGH46) was more rapid than that of the unconjugated drug CGH45.

There was a high degree of liver uptake of radiolabel when 2.3 μg of CGH46 were administered to male Wistar rats: 250 sec after dosing, 64% of the recovered radioactivity was recovered from the liver (Table 1). The relative radioactivity per gram of tissue was 5.2 times higher in liver as compared to whole blood, the only other tissue associated with high amounts of radiolabel. However, liver uptake of the conjugate was not significantly greater than uptake of the unconjugated ansamycin CGH45 ($P = 0.41$; unpaired Student's *t*-test), with 57% of recovered activity in the liver (Table 1) representing accumulation of radiolabel 3.8 times greater than that in blood. Hepatic accumulation of ASOR was

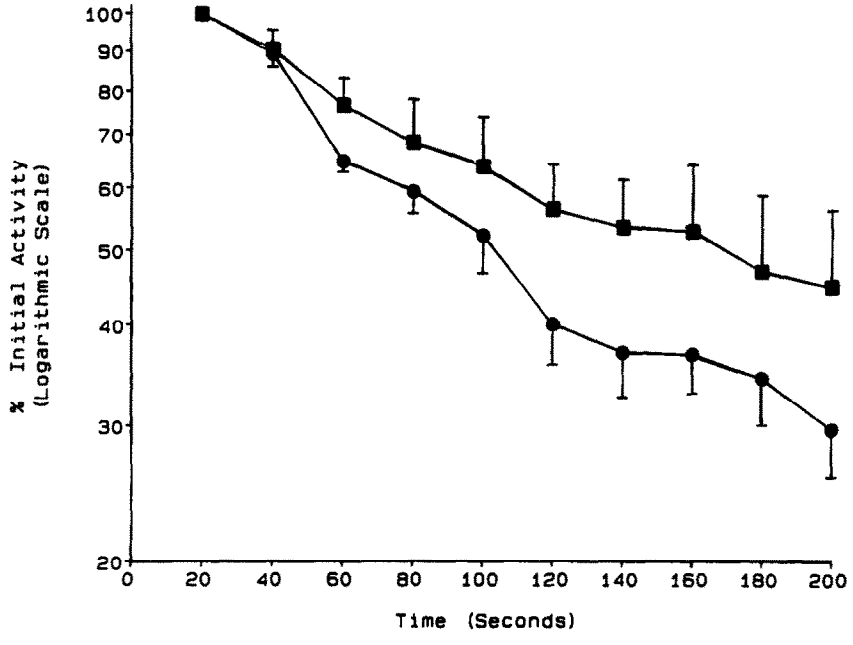

Fig. 2. Clearance of CGH45 (■) and CGH46 (●) from the blood stream of male Wistar rats following an i.v. administered dose of radiolabelled compound. % initial activity: radioactivity in the first blood sample withdrawn 15 sec of administration of the dose is taken as 100%.

Table 1. The recovery of radiolabel from various tissues after i.v. administration of compounds to male Wistar rats

Organ	CGH46 (N = 6)	CGH45 (N = 4)	Tris-gal-acetate (N = 5)	ASOR (N = 5)
Liver	63.9 ± 6.5	57.0 ± 6.9	2.2 ± 0.1	91.9 ± 4.3
Blood	18.1 ± 6.0	23.0 ± 4.2	38.5 ± 6.7	2.3 ± 4.3
Lungs	4.4 ± 1.4	3.9 ± 1.2	0.5 ± 0.1	0.1 ± 0.0
Kidneys	1.7 ± 0.2	0.9 ± 0.1	13.7 ± 1.3	0.4 ± 0.1
Spleen	0.6 ± 0.7	1.0 ± 0.2	0.1 ± 0.1	0.0 ± 0.0
Intestine	3.7 ± 2.1	2.2 ± 0.4	6.9 ± 1.2	1.5 ± 0.5
Skin	4.6 ± 3.2	2.6 ± 0.5	17.7 ± 2.2	1.3 ± 0.2
Muscle	2.4 ± 3.9	7.2 ± 1.7	18.4 ± 3.8	2.0 ± 0.3
Fat	0.4 ± 1.0	1.5 ± 0.9	1.7 ± 0.6	0.3 ± 0.1
Remaining organs	0.8	0.7	0.3	0.2
Total Recovery	88.0 ± 10.6	92.9 ± 16.9	94.6 ± 3.9	101.8 ± 4.7

Data represent % of radioactivity recovered (means ± 1 SEM) except total recovery values, which represent % of injected activity recovered. Animals were killed 250 sec after administration. Animals received approximately 10,000 dpm.

Table 2. Uptake of compounds by isolated perfused rat livers

Compound	Amount infused/min/g	Competitor (amount/min/g)	Uptake of compound		N
			Amount/min/g	% dose	
[^{125}I]ASOR	6.4	None	2.94 ± 0.48 fmol	49.36 ± 7.98	5
	6.4	GalNAc (2.8 μmol)	0.44 ± 0.14 fmol	6.42 ± 2.05	5
CGH46	11.4 nmol	None	1.61 ± 0.06 nmol	14.09 ± 0.49	6
	2.9 nmol	None	0.55 ± 0.09 nmol	19.64 ± 3.17	6
	2.9 nmol	GalNAc (2.4 μmol)	0.63 ± 0.08 nmol	20.45 ± 2.63	6
	2.9 nmol	ASF (9.4 nmol)	0.51 ± 0.09 nmol	19.00 ± 2.40	6
CGH45	2.3 nmol	None	0.21 ± 0.04 nmol	9.02 ± 1.60	6
Tris-gal-acetate	7.6 nmol	None	0.34 ± 0.23 nmol	4.43 ± 3.01	6
	7.6 nmol	GalNAc (2.3 μmol)	0.34 ± 0.12 nmol	4.52 ± 1.56	6

Results are expressed as means ± 1 SEM over the 5 min perfusion period.

high, with 92% of radioactivity recovered 250 sec after administration of 0.11 μg. In contrast, only 2.2% of the low molecular weight tris-gal-acetate conjugate (dose of 4.13 μg) was taken up by the liver over this time period (Table 1).

Distribution of radiolabel 30 min after dosing was also determined for CGH45 and CGH46. The patterns of distribution were comparable to those found after 250 sec. There was a slight reduction in label recovered from the liver: 51.5% for CGH45 (total recovery: 98.7 ± 3.4% of injected activity) and 53% for CGH46 (total recovery: 86.5 ± 6.3%), and an increase in the amount in the intestine (4.5% for CGH45 and 7% for CGH46).

Uptake of compounds by the isolated perfused rat liver

Results of experiments designed to determine the uptake of compounds by the isolated perfused Wistar rat liver are shown in Table 2. First pass extraction of ASOR, a ligand with a high affinity for the ASGP receptor [5], was almost 50% of the given dose; ASOR uptake was significantly reduced by the presence of GalNAc in the perfusion medium (87% uptake inhibition). First pass extraction of the conjugate CGH46 was approximately 20%, double the extraction rate of the unconjugated ansamycin CGH45. However, hepatic uptake of the conjugate was unaffected by addition to the perfusion medium of ASF or an approximately 800-fold molar excess of GalNAc (Table 2). The extraction of tris-gal-acetate was minimal and was unchanged by the addition of GalNAc.

Kinetic studies of the removal of compounds from the perfusion medium by isolated perfused liver (Fig. 3) showed that there was a constant rate of extraction during the course of the experiments, indicating no saturation of specific and non-specific uptake systems. There was no significant release from the liver of any of the perfused compounds either during the 5 min perfusion period (Fig. 3) or after termination of compound perfusion. For a period of 2–10 min after the cessation of compound perfusion, the concentrations of compounds in the perfusate collected from the livers were less than 2% of the administered concentrations for CGH45 and CGH46 and near background for the radiolabelled compounds.

DISCUSSION

It is evident from the pharmacokinetic and liver perfusion studies reported here that the tris-galactosyl-ansamycin conjugate CGH46 was rapidly

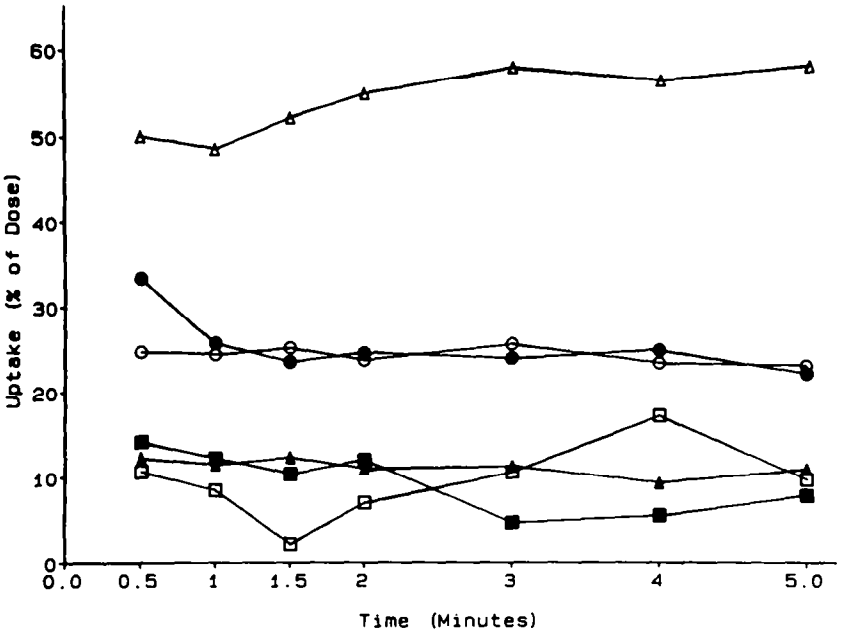

Fig. 3. Time course of removal of compounds from the perfusion medium of isolated perfused Wistar rat livers. Perfusion was non-recirculating and compounds were perfused over 5 min. Liver uptake is expressed as % of dose/g liver/min. Error bars were omitted for clarity; number of experiments for each compound is as given in Table 2. (△) ASOR; (▲) ASOR + GalNAc; (○) CGH46; (●) CGH46 + GalNAc; (■) CGH45; (□) tris-gal-acetate.

extracted from the circulation by the liver, the presumed site of pharmacological action. Liver uptake, however, was clearly not related to binding of the conjugate to the ASGP receptor, as neither ASF nor GalNAc had an effect on the uptake of CGH46 by the isolated perfused rat liver. Both ASF and GalNAc have been shown to compete for ASGP receptor binding sites and to reduce receptor-mediated ASGP uptake [4, 20]. In addition, the distribution of i.v. administered CGH45 in Wistar rats was not significantly different from that of the conjugate, suggesting that the properties of the drug and not the targeting moiety determined *in vivo* distribution of the conjugate. Furthermore, there was little difference between CGH45 and CGH46 in their capacity to reduce the levels of plasma cholesterol in Wistar rats (unpublished observations). Results obtained with tris-gal-acetate also support the contention that specific liver uptake is not facilitated by derivatization with monomeric tri-antennary galactosyl moieties.

In distribution studies, the initial time point of 4 min was chosen to allow for accumulation of endocytosed ASGP receptor ligands in the liver [4] and to avoid loss of radioactivity from cells as a result of rapid degradation and release of endocytosed conjugates [8]. In fact, the distribution of label appeared to change little over the period from 4 to 30 min except for a slight increase in radiolabel in the small intestine indicative of enterohepatic circulation of the conjugate or its metabolites. Such distribution profiles are compatible with established pharmacokinetic models for Rifampin, in which two interconnecting subsystems have been proposed [21].

There is ample evidence that small galactose- of GalNAc-terminated molecules are able to inhibit the ASGP receptor binding and internalization of a variety of macromolecular ligands [8, 22, 23], and we have demonstrated, in experiments to be reported elsewhere, that CGH46 is able to inhibit the binding of [^{125}I]ASOR to the HepG2 cell line. There is, however, scant information on the fate of bound low molecular weight ligands. Connolly *et al.* [8] showed that cluster glycosides containing either one, two or three galactosyl residues per ligand were able to bind to hepatocytes, and that receptor–ligand complexes were probably internalized but did not accumulate inside the cell. Instead, undegraded ligands were rapidly recycled back to the surface and released from the cells. It is surprising, therefore, that the triantennary galactose-derivatized ansamycin CGH46 showed no greater liver accumulation than the unconjugated ansamycin as ASGP receptor binding alone, even in the absence of internalization, would be expected to increase that proportion of the dose inititially (250 sec) associated with the liver. That this did not occur may reflect the inability of synthetic putative ligands to compete *in vivo* with a comparatively large circulating pool of natural ligands. Indeed, liver uptake of tris-gal-acetate was minimal (Tables 1 and 2) and, unsurprisingly, addition of GalNAc had no effect on the low rate of extraction by the perfused liver (Table 2).

It is unlikely that the apparent lack of specific uptake was due to saturation of ASGP receptor sites, since we were careful not to administer saturating doses. Also, it is well established that ASGP receptor-medicated uptake results in rapid loss of receptor sites from the cell membrane [5, 20]; therefore, receptor saturation in our liver perfusion system would probably result in a higher initial uptake followed by a progressive decrease. The

constant rate of uptake observed (Fig. 3) argues against receptor saturation, and validation of our assay systems with ASOR indicated uptake and inhibition patterns similar to those found by other workers [4, 22]. However, the experimental conditions were such that a very rapid (<30 sec) short circuit pathway involving transient internalization and release could have remained undetected.

Our results lend indirect support to the contention that interaction of galactosyl residues with the ASGP receptor, although necessary, is insufficient to elicit irreversible endocytosis of the ligand. It has been proposed that only multivalent ligands with appropriate determinant spacing will induce the conformational changes in the receptor which trigger endocytosis [5]. Thus, monomeric triantennary galactosyl conjugates would not accumulate inside hepatocytes *in vivo*; further attempts to use this pathway to target substances to the liver should focus on the likely requirements for appropriate spacing of multivalent galactosyl residues.

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