Biochemical Pharmacology, Vol. 45, No. 4, pp. 917–925, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

HEPATOCELLULAR UPTAKE OF PEPTIDES—II

INTERACTIONS BETWEEN HYDROPHILIC LINEAR RENIN-INHIBITING PEPTIDES AND TRANSPORT SYSTEMS FOR ENDOGENOUS SUBSTRATES IN LIVER CELLS

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(*Received* 1 *June* 1992; *accepted* 20 *November* 1992)

Abstract—To define the endogenous transport system responsible for the hepatocellular uptake of hydrophilic linear peptides, interactions between the cationic renin-inhibitor, [5(4-amino-piperidyl-1-carbonyl-L-2,6[^{3}H]phenyl-alanyl-β-alanyl(4*S*-amino-3*S*-hydroxy-5-cyclo-hexyl)-pentan-carbonyl-L-isoleucyl-aminomethyl-4-amino-2-methyl-pyrimidine-citrat] (code number EMD 56133; EMD, E. Merck, Darmstadt) and substrates of endogenous transport systems of liver cells were studied in isolated rat hepatocytes. EMD 56133 competitively inhibited the uptake of ouabain (K_i = 75 μM) and vice versa (K_i = 200 μM). In contrast, the sodium-dependent as well as the sodium-independent uptake of cholate and the total uptake of taurocholate were non-competitively blocked, whereas EMD 56133 decreased the uptake of the cyclosomatostatin 008 in an uncompetitive manner. EMD 56133 did not interfere with transport systems for monovalent organic cations, amino acids and long chain fatty acids. The uptake of rifampicin, however, was increased in the presence of EMD 56133. The transport of EMD 56133 was non-competitively inhibited by cholate (K_i = 126 μM) and taurocholate (K_i = 44 μM), and uncompetitively inhibited by the linear peptide EMD 51921. In contrast, the uncharged compound ouabain (K_i = 200 μM) and the bivalent organic cation *d*-tubocurarine (K_i = 370 μM) competitively inhibited the uptake of the renin inhibitor. Several substrates of other endogenous transport systems (e.g. bilirubin, cyclopeptides, monovalent cations, dipeptides, amino acids, fatty acids, hexoses) did not interfere with the transport of EMD 56133. Our results suggest that transport systems for bivalent organic cations or uncharged compounds (ouabain) are able to eliminate the linear hydrophilic peptide tested.

In the preceding paper we investigated the mechanism of the uptake of the linear hydrophilic cationic peptide EMD 56133 (E. Merck, Darmstadt†; [5(4-amino-piperidyl-1-carbonyl)-L-2,6[^{3}H]phenyl-alanyl-β-alanyl(4*S*-amino-3*S*-hydroxy-5-cyclo-hexyl)-pentan-carbonyl-L-isoleucyl-aminomethyl-4-amino-2-methyl-pyrimidine-citrat]) (renin-inhibitor) into isolated rat hepatocytes [1]. These studies indicated that the hydrophilic cationic peptide is transported into hepatocytes by a carrier-mediated mechanism which is energy dependent but not dependent on sodium. The membrane potential may be a driving force for the uptake of the cationic compound. The aim of the present study was to examine the physiological transport system which is responsible for the elimination of EMD 56133. We studied the interference of the hydrophilic renin-inhibitor EMD 56133 with endogenous transport systems of liver cells and *vice versa*. Several transport systems for endogenous substrates, which are also able to transport foreign compounds such as drugs or toxins [2] have been identified in liver cells. A transport system termed the "multispecific" bile acid transporter (MT) [3] has a very broad substrate specificity: steroids (bile acids, fusidic acid), organic anions [iodipamide, 4,4′-diisothiocyano-1,2-diphenylethan-2,2′-disulfonic acid (DIDS)], and hydrophobic peptides (phalloidin, antamanide, somatostatin analogs, hydrophobic linear renin-inhibitors) [3–5]. In contrast, the sodium-dependent taurocholate transporter seems to be more specific for conjugated bile acids since the K_i values for the inhibition of taurocholate uptake by foreign compounds are very high [6]. Besides sodium-dependent bile acid transport systems that are specific for conjugated or unconjugated bile acids, a sodium-independent transporter for all bile acids has been postulated [7, 8].

Furthermore the liver possesses transport systems for uncharged compounds (e.g. ouabain), organic anions (e.g. bilirubin) which also transport rifampicin, indocyanine green and bromosulfophthalein (BSP) [2], bivalent organic cations (e.g. vecuronium, *d*-tubocurarine) [9], monovalent organic cations (e.g. thiamine hydrochloride), amino acids and long-chain fatty acids [10].

The uptake of typical substrates of the above transport systems was measured in the presence of EMD 56133. In addition, the uptake of [^{3}H]EMD 56133 was measured in the presence of typical substrates of the endogenous transport systems.

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† Abbreviations: EMD, E. Merck, Darmstadt; PBS, phosphate-buffered saline; DIDS, 4,4′-diisothiocyano-1,2-diphenylethan-2,2′-disulfonic acid; BSP, bromosulfophthalein; 008, cyclosomatostatin.

MATERIALS AND METHODS

Materials

The linear peptides [^{3}H]EMD 56133/EMD 56133 and [^{3}H]EMD 51921/EMD 51921 were gifts from Merck (Darmstadt, F.R.G.). [^{14}C]Cholic acid sodium salt (sp. act. 2.07 GBq/mmol) and [^{3}H]ouabain (sp. act. 777 GBq/mmol) were purchased from Amersham Buchler (Braunschweig, F.R.G.). [^{3}H]-Taurocholic acid sodium salt (sp. act. 222 GBq/mmol) and [^{14}C]oleic acid (sp. act. 2.1 GBq/mmol) were purchased from Du Pont-De Nemours (Dreieich, F.R.G.). The following compounds were kindly provided as gifts: [^{14}C]thiamine hydrochloride (sp. act. 851 MBq/mmol) from Dr A. Hahn (Gießen, F.R.G.); [^{3}H]vecuronium from Dr D. K. F. Meijer (Groningen, Netherlands); [^{3}H]serine (sp. act. 32.5 GBq/mmol) from Dr W. Föllmann (Gießen, F.R.G.); [^{14}C]rifampicin (sp. act. 42.8 MBq/mmol) from Ciba-Geigy (Basel, Switzerland); [^{3}H]cyclo-somatostatin (008) (sp. act. 10.86 GBq/mmol) from Prof. Dr H. Kessler (Frankfurt, M., F.R.G.).

All other chemicals were of the highest purity grade available commercially.

To stabilize the suspension of oleate in the aqueous incubation medium, it was bound to albumin. For that [^{14}C]oleic acid–ethanol solution was dried under a N_2 gas stream and then mixed with oleic acid. The compounds were dissolved in 0.1 N NaOH at 37° and were added to a solution of bovine serum albumin in phosphate-buffered saline (PBS) in a ratio of 1:1 (mol:mol).

The Tyrode buffer was composed of 137 mM NaCl, 2.7 mM KCl, 1.05 mM $MgCl_2$, 1.8 mM $CaCl_2$, 12 mM $NaHCO_3$, 5.55 mM glucose and 0.42 mM NaH_2PO_4.

In the lithium-Tyrode buffer NaCl and Na_2CO_3 were replaced by LiCl and Li_2CO_3, respectively. The pH of all solutions was adjusted to 7.4 with 1 N HCl at 37°.

Methods

Isolation of rat liver cells. The hepatocytes were prepared from male Wistar rats (body weight 200–260 g) by the method of Berry and Friend [11]. After 30 min of equilibration in Tyrode buffer, pH 7.4 at 37° in carbogene atmosphere (95% O_2, 5% CO_2), the cell-viability was tested by the Trypan blue exclusion test [12]. The experiments were performed within 2 hr after isolation with a hepatocyte suspension containing more than 80% viable cells under incubation conditions as mentioned above.

Uptake of bile acids, organic anions, organic cations, steroids, peptides, amino acids and fatty acids in the presence of EMD 56133. For all experiments, a liver cell suspension with 2×10^6 cells/mL was used; the suspension contained 4 mg cell protein/mL as determined with Bradford reagent [13].

One milliliter of the hepatocyte suspension was preincubated for 30 sec with 25, 75 or 126 μM of EMD 56133 (pH 7.4). Aliquots (100 μL) were taken at 15, 45, 75, 105 and 135 sec after the addition of 17 μM [^{14}C]cholic acid, 4.8 μM [^{3}H]taurocholate, 15/100 μM [^{14}C]rifampicin, 16 μM [^{3}H]vecuronium, 27.4 μM [^{3}H]ouabain, 1.1 μM [^{3}H]008, 2.3 μM [^{3}H]-EMD 51921, 1 mM [^{3}H]serine. Samples for the uptake of 3 μM [^{14}C]thiamine hydrochloride were taken 15, 30 and 60 sec after addition; the uptake of 17 μM [^{14}C]oleic acid was determined 15 and 30 sec after the mixture was added to the hepatocytes. The 100 μL aliquots were pipetted into Microtest^R-tubes. The uptake of the substances was stopped by silicone layer centrifugation [14] using a Beckman Microfuge B. The amount of substance taken up into the filtered cells was determined by radioactivity measurements in the sediment fraction by liquid scintillation counting.

Uptake of [^{3}H]*EMD* 56133 *in the presence of typical substrates of physiological transport systems.* One milliliter of the hepatocyte suspension was incubated for 30 sec with different concentrations of the substrates (Table 3). A mixture of 2.14 μM [^{3}H]-EMD 56133 and different concentrations of unlabeled EMD 56133 dissolved in 50 μL PBS buffer was then added.

Uptake kinetics. Different concentrations of the unlabeled substrates were incubated for 30 sec with 1 mL liver cell suspension. A constant concentration of the labeled substance was mixed with one of five different concentrations of unlabeled substrate and added to the incubation mixture. In the case of [^{3}H]-008, only the labeled substrate was used. The initial uptake rates and the concentration of these substrates were plotted according to Lineweaver and Burk [15], Woolf [16], Hofstee [17] and Cornish-Bowden [18] to determine kinetic parameters and the type of transport inhibition (only Cornish-Bowden plots are shown); the K_i was determined according to Dixon [19].

Uptake kinetics in sodium-free Tyrode buffer. After the hepatocytes were diluted to a concentration of 2×10^6 cells/mL Na^+-Tyrode, the cells were washed twice in Na^+-free Tyrode buffer for 2–3 sec in an Eppendorf centrifuge. The pellets were dissolved in the same buffers. After 10 min of equilibration time the compounds were added; 30 sec later the uptake of the mixture of labeled and unlabeled substrate was measured.

Statistical methods. The initial velocity of the uptake was calculated from the uptake vs time graphs, the IC_{50} values were determined from the graph of the percentage inhibition vs the log concentration. All experiments were performed using triplicate cell preparations, the average of the three values were used for further calculations. The plots mentioned above were calculated by linear regression analysis, the IC_{50} values by logarithmic regression analysis. Statistical significance ($\alpha = 0.01$) was assessed with the program SPSS/PC$^+$. The results were significant when $P \leq 0.01$.

RESULTS

Influence of the peptides on cell viability

The viability of the liver cells was tested by Trypan blue exclusion after the addition of several concentrations of EMD 56133. None of the added concentrations caused a significant increase in cell mortality. After incubation of the cells for 2 hr with the highest concentration chosen (630 μM EMD 56133) 80% of the cells excluded the dye.

Table 1. Influence of EMD 56133 on the hepatocellular uptake of substrates of endogenous transport systems of liver cells

Transport systems	Substrates	Concn (μM)	% maximal inhibition	IC_{50} (μM)	P
Bile acids	Cholate	(17)	57.9	308	0.0001
	Taurocholate	(4.8)	85.2	130	0.0001
Neutral compounds	Ouabain	(27.4)	73.6	75	0.0002
Cyclopeptides	008	(1.1)	53.7	87	0.0013
Linear peptides	EMD 51921	(2.3)	14.8	867	0.0001
Anionic compounds	Rifampicin*	(15)	330.7*	—	0.0071
		(100)	338.9*	—	0.0026
Cationic compounds	Vecuronium†	(16)	—	—	0.0003
	Thiamine hydrochloride‡	(3)	—	—	0.0001
Amino acids	Serine	(1000)	—	—	0.0146
Fatty acids	Oleate	(17)	—	—	0.0293

— No inhibition of the uptake.
* Increase of the uptake.
† Bivalent.
‡ Monovalent.
Hepatocytes were incubated with different concentrations of EMD 56133 before the substrates were added. The uptake of the substrates was measured as described in Materials and Methods. The initial uptake was determined from the slope of the linear part of the uptake.
The IC_{50} values correspond to the concentration of EMD 56133 needed for 50% inhibition of the uptake of the indicated concentrations of the substrates. IC_{50} values were determined by logarithmic regression analysis.
N = 3.
P = significance of the inhibition at $\alpha = 0.01$.

The influence of EMD 56133 on the uptake of substrates of endogenous transport systems into isolated hepatocytes: multispecific bile acid transporters. EMD 56133 decreased the initial uptake of cholate [7, 8] and taurocholate [8], the cyclosomatostatin 008 [20–22] and the linear peptide EMD 51921 [4, 5] in a concentration-dependent manner (Table 1, Fig. 1c).

Transporter for organic anions. The uptake of the organic anion rifampicin [23] (a substrate of the bilirubin transporter), on the other hand, increased in the presence of EMD 56133 (Fig. 2a and b).

Transporter for uncharged compounds. EMD 56133 inhibited the initial uptake of ouabain [24]. The affinity of EMD 56133 to this transport system was high ($IC_{50} = 75\ \mu M$).

Kinetics of the inhibition of the uptake of certain substrates of endogenous transport systems in the presence of EMD 56133

Bile acid transporters. As mentioned above, multispecific bile acid transporters [3, 6] have been shown to be responsible for the rapid elimination of hydrophobic renin inhibitors. Since the hydrophilic inhibitor EMD 56133 also inhibits these transport systems, it was of interest to determine the characteristics of their uptake inhibition. For this reason the uptake of the physiological substrates cholate and taurocholate, of the xenobiotic 008, and of EMD 51921 was measured in the presence of EMD 56133.

The linear peptide EMD 56133 inhibited the uptake of both cholate and taurocholate in a non-competitive manner (Figs 1a and 3a).

The uptake of the cyclic peptide 008 was inhibited in an uncompetitive manner (Fig. 1b), whereas EMD 51921 was competitively inhibited (Fig. 1c).

The uptake of cholate into hepatocytes is 50% Na^+ dependent and 50% Na^+ independent [8]. To determine which of the two cholate-carriers was non-competitively inhibited by EMD 56133, the hepatocellular uptake of cholate in the presence of EMD 56133 was measured in Na^+-free lithium-Tyrode.

The Na^+-independent uptake of cholate in Li^+-Tyrode was also inhibited by EMD 56133 in a non-competitive manner (Fig. 3b).

Transporter for uncharged compounds. In the case of ouabain uptake the inhibition was competitive ($K_i = 75\ \mu M$). The kinetic constants calculated are shown in Table 2. EMD 56133 has a high affinity to the transport system of ouabain ($K_i = 75\ \mu M$), whereas the affinity is low to bile acid transporters ($K_i = 141$–$334\ \mu M$) and to the transport system for hydrophobic linear peptides ($K_i = 890\ \mu M$).

Unchanged uptake of thiamine hydrochloride, serine and oleic acid in the presence of EMD 56133

Since further endogenous transport systems which may be able to transport the linear peptide have been described for liver cells, we studied the uptake of 3 μM [^{14}C]thiamine hydrochloride [25], 1 mM [^{3}H]serine [26], and 17 μM albumin-bound [^{14}C]-oleic acid [10] in the presence of EMD 56133. The concentrations used corresponded to the K_m values reported for the substrates. The initial velocities, which were measured in our experiments, were not

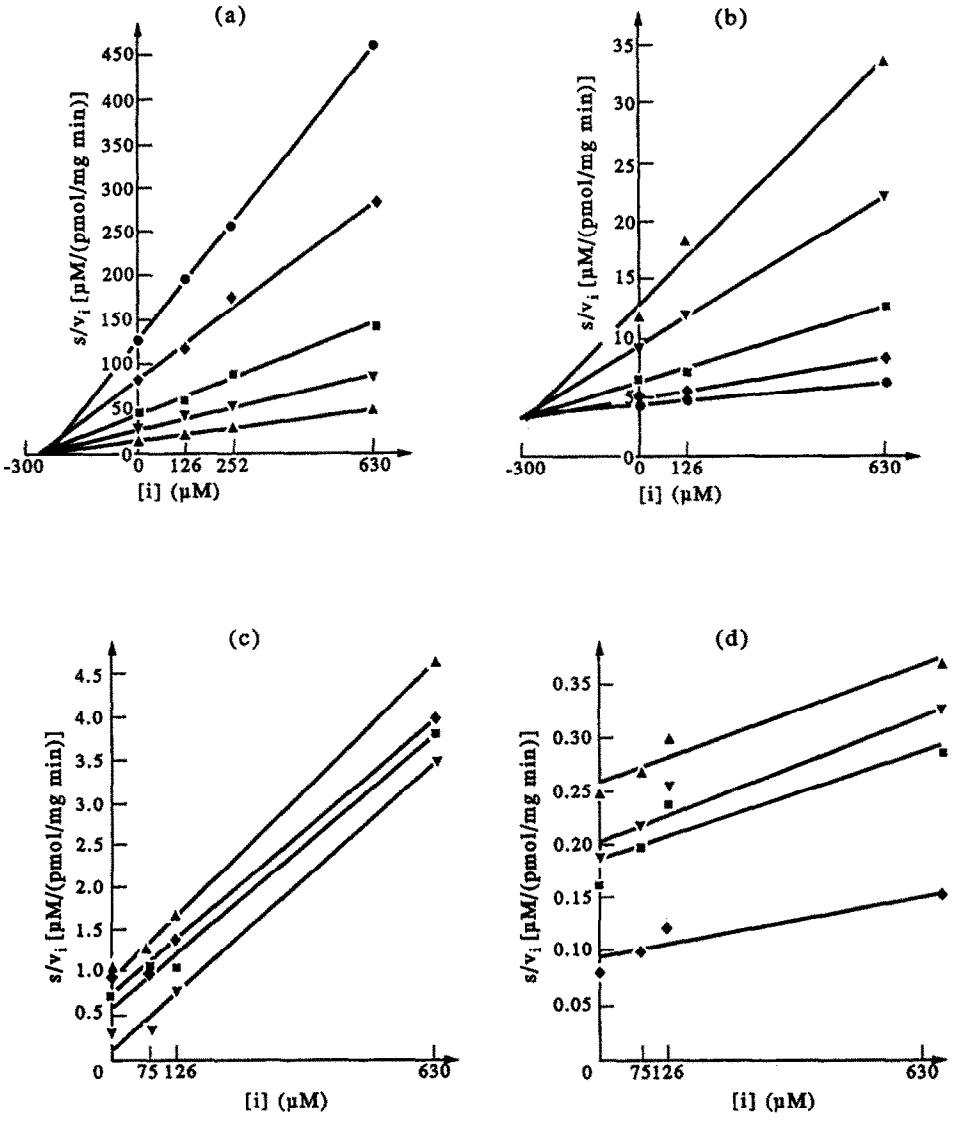

Fig. 1. (a) Cornish-Bowden plot of the taurocholate uptake in the presence of EMD 56133. The uptake of increasing concentrations [4.8 (▲), 9.7 (▼), 19 (■), 39 (◆) or 97 μM (●)] of [^{3}H]taurocholate was measured 30 sec after the addition of 0 (control), 126, 252 or 630 μM EMD 56133. Initial uptake was calculated between 15 and 135 sec. K_i = 151 μM; $r \geq 0.987$; N = 3. (b) Cornish-Bowden plot of the uptake of 008 in the presence of EMD 56133. The uptake of 0.8 (●), 1.5 (◆), 7.5 (■), 15 (▼) and 150 μM (▲) [^{3}H]008 was measured after 30 sec incubation of the hepatocytes with 0 (control), 126 or 630 μM EMD 56133. The initial uptake was calculated between 15 and 135 sec. K_i = 450 μM; $r \geq 0.96$; N = 3. (c) Cornish-Bowden plot of the uptake of ouabain in the presence of EMD 56133. The uptake of 49.4 (▼), 247 (■), 370 (◆) and 494 μM (▲) [^{3}H]ouabain was measured after 30 sec incubation of the hepatocytes with 0 (control), 75, 126 or 630 μM EMD 56133. The initial uptake was calculated between 15 and 135 sec. K_i = 75 μM; $r \geq 0.996$; N = 3. (d) Cornish-Bowden plot of the hepatocellular uptake of the hydrophobic linear peptide EMD 51921 in the presence of EMD 56133. The uptake of increasing concentrations 0.664 (◆), 3.3 (■), 6.6 (▼) and 13.2 μM (▲) of [^{3}H]EMD 51921 was measured 30 sec after the addition of 0 (control), 75, 126 or 630 μM EMD 56133. Initial uptake was calculated between 15 and 135 sec. K_i = 890 μM; $r \geq 0.918$; N = 3.

influenced by EMD 56133 within a concentration range of 25–126 μM (Table 1).

Influence of substrates of endogenous transport systems of liver cells on the uptake of EMD 56133

Multispecific bile acid transporter (MT) and sodium–taurocholate cotransporter (Table 3). Cholate and taurocholate inhibited the uptake of the hydrophilic linear peptide EMD 56133 in a non-competitive manner (Fig. 4a and c). The uptake of EMD 56133 was also inhibited non-competitively by cholate in a Na^+-free Tyrode buffer (Fig. 4b). Linear

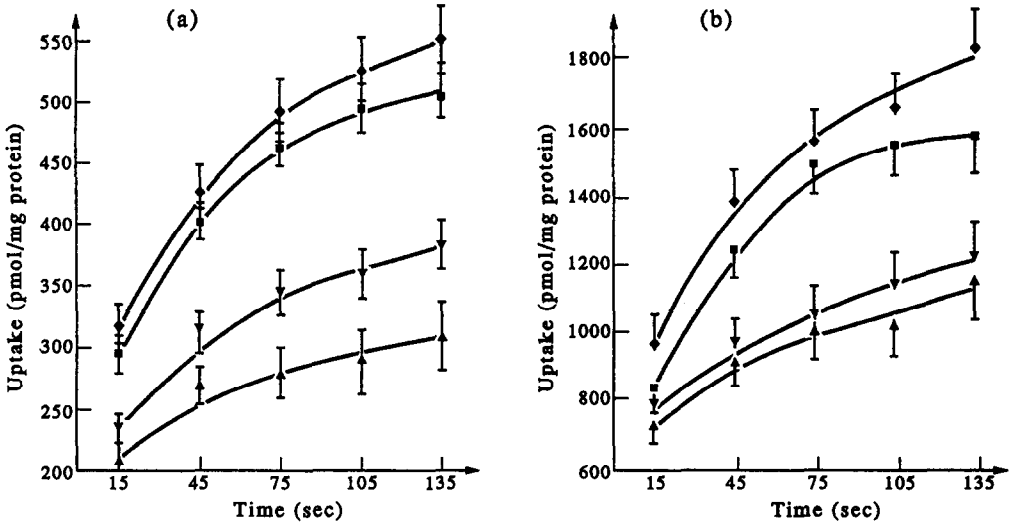

Fig. 2. Influence of EMD 56133 on the uptake of rifampicin. Hepatocytes were preincubated with increasing concentrations of EMD 56133 [control (▲), 25 (▼), 75 (■) or 126 μM (◆)] for 30 sec before 15 (a) or 100 μM [^{14}C]rifampicin (b) was added. $P_{15} = 0.0071$; $P_{100} = 0.0026$; N = 3; mean ± SD.

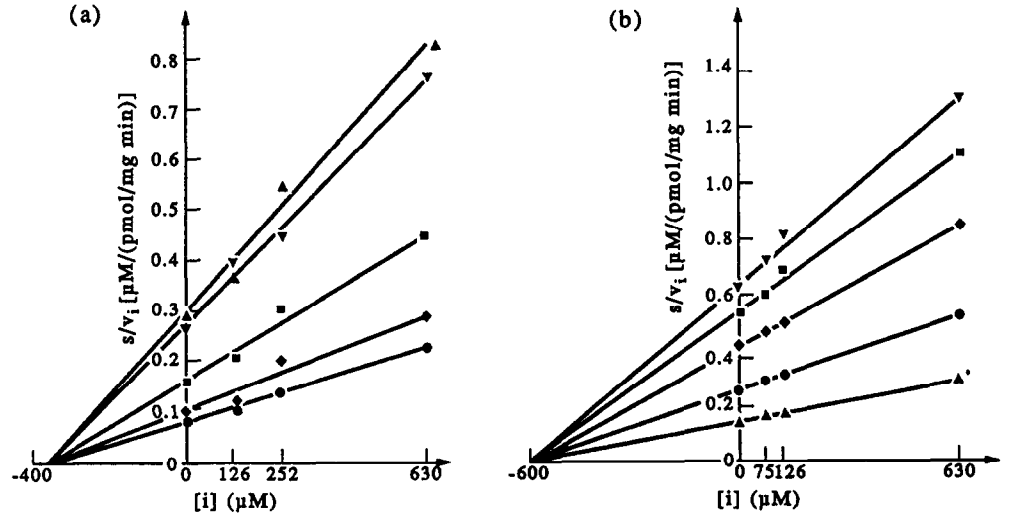

Fig. 3. (a) Cornish-Bowden plot of the cholate uptake in the presence of EMD 56133. One milliliter of liver cell suspension was incubated for 30 sec with 0 (control), 126, 252 or 630 μM EMD 56133 prior to the addition of different concentrations [6 (●), 12 (◆), 24 (■), 48 (▲) or 115 μM (▼)] of [^{14}C]-cholate. Initial uptake was calculated between 15 and 135 sec. $K_i = 334$ μM (according to Dixon); $r \geq 0.986$; N = 3. (b) Cornish-Bowden plot of the uptake of cholate in the presence of EMD 56133 in Na^+-free medium. Liver cell suspension (2×10^6 cells/mL) has been washed twice in Li^+-Tyrode at 30 *g*. After 10 min equilibration time the cells were incubated for 30 sec in 0, 75, 126 or 630 μM EMD 56133 and the uptake of different concentrations [12 (▲), 24 (●), 115 (◆), 230 (■) or 576 μM (▼)] [^{14}C]cholate was measured. The initial velocities were determined from values between 15 and 135 sec. $K_i = 600$ μM; $r \geq 0.96$; N = 3.

and cyclic peptides: both 008 and EMD 51921 inhibited the uptake of EMD 56133 in an uncompetitive manner (Table 4). The transport constants calculated from the plots mentioned above are shown in Table 4.

Other transport systems (Table 2). Bilirubin carrier: the organic anions BSP, rifampicin and rose bengal did not inhibit peptide uptake (Table 3). Organic cations: neither the hydrophobic bivalent organic cation vecuronium nor the monovalent cations thiamine hydrochloride and *n*-methylnicotinamide inhibited the uptake of EMD 56133 (Table 3). Only

Table 2. Kinetic constants of the plots of Figs 3 and 1

Substrate	K_i (μM)	K_m (μM)	V_{max} (pmol/mg min)	Type of inhibition
Taurocholate	151	19	352	Non-competitive
Cholate	334	26	667	Non-competitive
Cholate (Li^+)	600	133	1174	Non-competitive
008	450	10	2.2	Uncompetitive
EMD 51921	890	3.5	51	Competitive
Ouabain	75	18	431	Competitive

The initial uptake of increasing concentrations of [^{14}C]cholate (6–115 μM), [^{14}C]cholate (12–576 μM) in Na^+-free Tyrode, [^{3}H]taurocholate (4.8–97 μM), [^{3}H]008 (0.8–150 μM), [^{3}H]-EMD 51921 (0.664–13.2 μM) or [^{3}H]ouabain (49.4–494 μM) was measured 30 sec after the addition of different concentrations of EMD 56133 (0–630 μM).

The initial uptake rates and the concentrations of the substrates were plotted according to Lineweaver and Burk [15], Woolf [16], Hofstee [17] and Cornish-Bowden [18]; the K_i was determined according to Dixon [19].

N = 3.

Table 3. Uptake of EMD 56133 into liver cells in the presence of representatives of different transport systems

Transport system	Inhibitor	Concn (μM)	P	IC_{50} (μM)	% Max. inhibition
Bile acids	Cholate	(5.8/115/230)	<0.0001	30	86.5
	Taurocholate	(19/97/194)	<0.0001	28	84.1
Neutral compounds	Digitoxin	(50/100/200)	=0.0221	—	—
	Ouabain	(2/13/68)	=0.0007	7.6	60.1
Cyclopeptides	008	(25/125/625)	=0.0081	511	52.3
Linear peptides	EMD 51921	(13.3/66.4/133)	=0.0054	393	41.7
Anionic compounds	Iodipamide	(17.5/44/88)	=0.5117	—	—
	Rifampicin	(28/139/278)	=0.0941	—	—
	Rose bengal	(40/400)	=0.1535	—	—
	BSP	(6/12/36)	=0.0571	—	—
Cationic compounds	Thiamine hydrochloride†	(59/296/1482)	=0.0489	—	—
	Vecuronium*	(78/157/314)	=0.0661	—	—
	Choline	(100/200/1000)	=0.1616	—	—
	n-Methylnicotinamide†	(100/200/1000)	=0.0712	—	—
	d-Tubocuranine	(100/200/1000)	<0.0001	370	62.3
Nucleosides	Uridine	(100/200/1000)			
	Adenosine	(100/200/1000)	≤0.2314	—	—
Amino acids	Serine	(500/1000/1500)		—	—
	AIB	(5000/10,000/20,000)	≤0.2247	—	—
	Cysteine	(5000/10,000/20,000)		—	—
	Alanine	(5000/10,000/20,000)		—	—
β-Amino acids	Methionine	(5000/10,000/20,000)			
	Phenylalanine	(5000/10,000/20,000)			
	Taurine	(5000/10,000/20,000)	=0.3134	—	—
Dipeptides	Cystine	(500/10,000/20,000)			
	Glycinoglycine	(5000/10,000/20,000)	—	—	
	Captopril	(10/20/40)	≤0.1715	—	—
Hexoses/ pentoses	Fructose	(5.5 mM)			
	Glucose	(5.5 mM)	≤0.1985	—	—
	Rhamnose	(5.5 mM)			
	Galactose	(5.5 mM)		—	—
Fatty acids	Oleate	(17/34/51)	=0.0211	—	—

* Bivalent.

† Monovalent.

— No inhibition.

Hepatocytes were preincubated with three concentrations of each substrate 30 sec before 75 μM [^{3}H]EMD 56133 was added. Initial velocity and IC_{50} values were determined as described in Table 2.

N = 3.

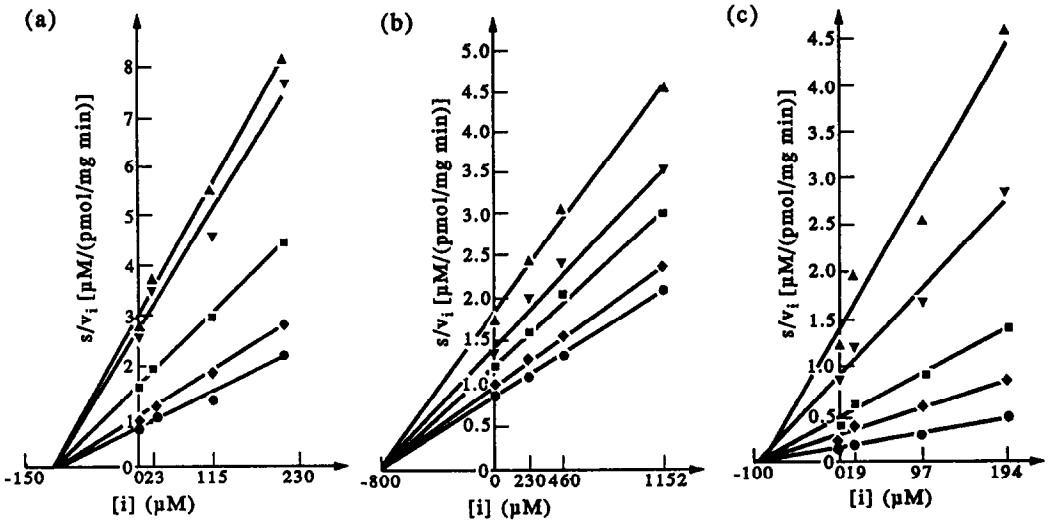

Fig. 4. Cornish-Bowden plots of the uptake of EMD 56133 in the presence of cholate in Na^+-Tyrode (a) and in Li^+-Tyrode (b) and in the presence of taurocholate (c). The uptake of 6.3 (●), 25 (◆), 75 (■), 126 (▼) or 630 μM (▲) of [^{3}H]EMD 56133 was measured 30 sec after the addition of: (a) 0 (control), 23, 115 or 230 μM cholate; K_i = 126 μM; $r \geq 0.989$; N = 3. (b) 0 (control), 230, 460 or 1152 μM of cholate (the cells were washed and incubated in Li^+-buffer as described in Materials and Methods); K_i = 415 μM; $r \geq 0.989$; N = 3; (c) 0 (control), 19, 97 or 194 μM taurocholate; K_i = 44 μM; $r \geq 0.961$; N = 3.

Table 4. Kinetic data of the EMD 56133 transport in the presence of bile acids, tubocurarine, 008, EMD 51921, and ouabain

Inhibitor	K_i Dixon (μM)	K_i Cornish-Bowden (μM)	Type of inhibition
Taurocholate	44	100	Non-competitive
Cholate	126	145	Non-competitive
Cholate (Li^+)	415	800	Non-competitive
008	110	80	Uncompetitive
EMD 51921	80	90	Uncompetitive
Ouabain	200		Competitive
d-Tubocurarine	370		Competitive

The initial uptake of increasing concentrations of [^{3}H]EMD 56133 (6.3–630 μM) was measured 30 sec after the addition of four increasing concentrations of cholate, taurocholate, ouabain, tubocurarine, 008 and EMD 51921. The initial uptake rates and the concentrations of the substrates were plotted according to Lineweaver and Burk [15], Woolf [16], Hofstee [17] and Cornish-Bowden [18]; the K_i was determined according to Dixon [19].

d-tubocurarine, a hydrophilic bivalent organic cation, inhibited the uptake of the peptide in a concentration-dependent manner (Table 3, Fig. 5). The type of transport inhibition turned out to be competitive (Table 4). Uncharged compounds: the uncharged steroid ouabain inhibited the uptake of the renin inhibitor competitively (K_i = 200 μM) (Fig. 6). Amino acids, β-amino acids and dipeptides: none of the compounds (see Table 3) blocked the uptake of the peptide. Hexose-carrier: fructose, glucose, galactose and rhamnose did not influence the peptide uptake (Table 3). Nucleosides: uridine and adenosine did not influence the transport of the renin inhibitor in any way (Table 3). Fatty acids: oleic acid had no influence on the uptake of EMD 56133 into liver cells (Table 3).

DISCUSSION

The short plasma half-life of hydrophobic linear renin-inhibiting peptides is due to their high affinity to a multispecific bile acid transporter [4, 5, 20] in the basolateral plasma membrane of liver cells. In contrast, hydrophilic linear peptides, which are also eliminated via the biliary pathway have a 200-fold lower affinity to the multispecific bile acid transporter [1]. Bile acids (cholate and taurocholate) and EMD 56133 are mutual non-competitive transport inhibitors. Hydrophilic peptides, therefore, are not substrates of bile acid carriers. Although the uptake of a hydrophobic linear peptide, EMD 51921 [4, 5], was competitively inhibited in the presence of EMD 56133, mutual inhibition studies showed that EMD

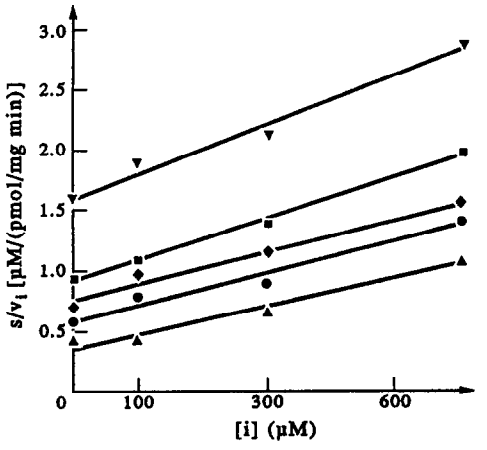

Fig. 5. Cornish-Bowden plot of the uptake of EMD 56133 in the presence of *d*-tubocurarine. The uptake of 6.3 (▲), 25 (●), 75 (◆), 126 (■) or 630 μM (▼) [^{3}]EMD 56133 was measured 30 sec after the addition of increasing concentrations (0–600 μM) of *d*-tubocurarine. K_i = 370 μM; $r \geq 0.986$; N = 3.

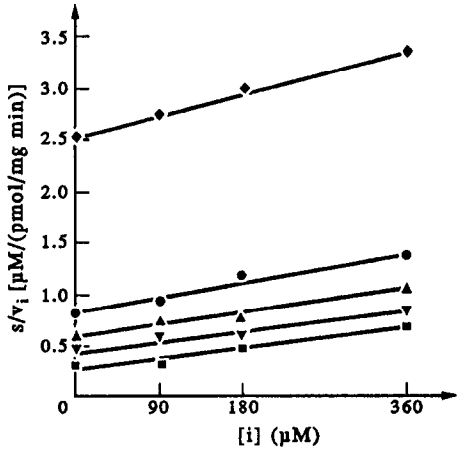

Fig. 6. Cornish-Bowden plot of the uptake of EMD 56133 in the presence of ouabain. The uptake of 6.3 (■), 25 (▼), 75 (▲), 126 (●) or 630 μM (◆) [^{3}H]EMD 56133 was measured 30 sec after the addition of increasing concentrations (0–360 μM) of ouabain. K_i = 200 μM; $r \geq 0.973$; N = 3.

51921 uncompetitively blocked the uptake of the hydrophilic peptide EMD 56133 (Table 4). Since only mutual competitive transport inhibition is indicative of a common transport system hydrophilic and hydrophobic peptides appear to be transported via different pathways.

EMD 56133 is a potent inhibitor of the uptake of the uncharged cardiac glycoside ouabain [24, 27, 28]. The K_i value is in the range of 75 μM. Ouabain transport was inhibited competitively by the peptide. Conversely, EMD 56133 uptake is also competitively inhibited by ouabain. The competitive type of inhibition suggests that a transport system for ouabain is a possible carrier for the linear peptide. In addition, ouabain competitively inhibits the uptake of the bivalent organic cation *d*-tubocurarine [29], which in turn competitively inhibits the uptake of EMD 56133. Since the hydrophilic peptide tested is cationic under physiological conditions (pK 5.9 and 8.9) it seems that a carrier for uncharged and bivalent cationic compounds may be able to translocate EMD 56133.

The question arises as to whether transport systems for uncharged compounds and bivalent organic cations can be responsible for the uptake of EMD 56133.

Hepatic uptake mechanisms for drugs and xenobiotics are often classified according to their charge i.e. transport systems for cationic, uncharged or anionic compounds [23, 29]. Several transport systems have been proposed for the different classes of compounds [29]. It is difficult to evaluate which of the transport systems is involved in the uptake of a given compound by kinetic studies alone. In a physiological milieu, organic compounds can be present in more than one form. Organic cations may form complexes as ion pairs with endogenous inorganic or organic counter ions and present themselves to the liver as electroneutral compounds [30, 31]. The model compound for the characterization of the hepatocellular uptake of uncharged compounds is ouabain [24]. Ouabain and EMD 56133 are mutual competitive transport inhibitors. Model compounds for the uptake of bivalent organic cations are the muscle relaxants vecuronium [32, 33] and *d*-tubocuranine [29]. Both compounds differ in their hydrophilicity, *d*-tubocurarine being more hydrophilic. Whereas the hydrophobic vecuronium reduced the association of EMD 56133 to the liver cell membrane without effecting its transport, the hydrophilic compound *d*-tubocurarine competitively blocked EMD 56133 uptake. Mutual competition studies were not possible because *d*-tubocurarine was not available in radiolabeled form. The transport system involved in the uptake of the peptide tested appears only to accept hydrophilic compounds. Other hydrophilic monovalent organic cations e.g. thiamine hydrochloride [25] and *n*-methylnicotinamide had no influence on the uptake of EMD 56133 ruling out the importance of their carriers for the uptake of EMD 56133.

Our studies suggest that the liver possesses several transport systems that are capable of eliminating peptides. The physicochemical properties (hydrophilic/hydrophobic balance) of the peptides determine the manner of their elimination and the type of physiological transporter used. Further studies e.g. expression cloning are needed to isolate the involved carrier protein and to answer the question concerning the physiological substrate of the carrier for hydrophilic peptides.

Acknowledgements—The authors wish to thank Dr B. Boschek and Dr J. Harting for critically reading the manuscript, Dr C. Schmitges for valuable discussion, Dr P. Raddatz and Dr F. Faro (E. Merck, Darmstadt, F.R.G.) for the synthesis of the peptides. This work was supported by the Deutsche Forschungsgemeinschaft SFB 249.

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