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Biochimica et Biophysica Acta 1196 (1994) 209–217

BBA Biochimica et Biophysica Acta

ATP-dependent transport of the linear renin-inhibiting peptide EMD 51921 by canalicular plasma membrane vesicles of rat liver: evidence of drug-stimulatable ATP-hydrolysis

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Received 28 March 1994; revised 30 August 1994

Abstract

Certain peptide drugs, such as the linear hydrophobic renin-inhibitor EMD 51921, are rapidly eliminated via the bile. At the sinosoidal membrane of liver cells EMD 51921 is taken up via a sodium-independent carrier-mediated mechanism, competing for the uptake of bile acids. Until now, the mechanisms of biliary excretion of EMD 51921 were unknown. In this study we describe an ATP-dependent transport system for the enzymatically and metabolically stable hydrophobic linear renin-inhibiting peptide EMD 51921. The ATP-dependent uptake into the osmotic reactive intravesicular space is saturable (K_m 12 μM, V_{max} 663 pmol/min per mg protein), temperature dependent and specifically requires ATP. Transport is inhibited by vanadate but not by ouabain, EGTA or NaN_3, and does not function in basolateral plasma membrane vesicles. Transport is not altered in canalicular membrane vesicles isolated from Tr^- rats lacking the canalicular ATP-dependent transport of cysteinyl leukotrienes and related anions. Transport is inhibited by taurocholate, a typical substrate of the canalicular ATP-dependent bile acid transporter, but also by vincristine and daunomycin, substrates of P-glycoproteins. EMD 51921, however, only inhibits the uptake of taurocholate, whereas the transport of daunomycin is not influenced. Taurocholate and EMD 51921 are mutually non- or un-competitive transport inhibitors. Incubation of rat liver canalicular membranes with micromolar concentrations of EMD 51921 resulted in a 1.8–2.5-fold increase in the rate of ATP-hydrolysis. In contrast, ATP-hydrolysis was not affected by fragments of the peptide that are not transported in an ATP-dependent manner. The apparent K_m value (EMD) for ATP-hydrolysis is 68 μM. V_{max} is 0.032 U/mg protein. ATPase activity is pH dependent. Stimulation of ATP-hydrolysis is inhibited by vanadate, NEM, hydroxymercuribenzoate and ascorbate, but is not affected by ouabain, EGTA or NaN_3. EMD 51921 does not stimulate the ATPase activity of the Na^+/K^+-ATPase isolated from kidney medulla. The EMD-stimulatable ATPase seems to be distinct from the glutathione-*S*-conjugate stimulatable ATPase and the mdr 1a/b gene products and differs in its characteristics from that of the canalicular ecto-ATPase.

Keywords: ATP hydrolysis; ATP-dependent transport; Peptide; Canalicular transport; (Rat liver)

1. Introduction

Although many efforts have been undertaken to develop drugs with peptide structure [1,2], only a few compounds are in clinical use [3]. This is the result of the short plasma half-life of the enzymatically stable peptides due to rapid biliary elimination [4]. Knowledge of the process of such hepatic uptake and secretion and their characteristics is limited [1–4]. Several studies on hepatocellular uptake mechanisms of peptide-drugs revealed that carrier-mediated mechanisms are responsible for their rapid extraction from portal blood [5–13]. Hydrophobic peptides such as the linear renin-inhibiting peptide EMD 51921 enter liver cells by a carrier-mediated mechanism [6,7], which is not dependent on the presence of sodium ions [7], but is inhibited by decreasing the intracellular ATP content [7]. By mutually competitive transport inhibition studies, the physiological substrates of this sinusoidal pseudopeptide transporter have been found to be bile acids [6]. 80% of EMD 51921 is excreted into the bile in an unmetabolized form (Schmitges, C., Merck-Darmstadt, unpublished results). The mechanism of the excretion process at the canalicular pole of the cell, however, is unknown.

Several transport systems have been described at the canalicular domain of liver cells (for reviews see [14–16]).

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SSDI 0005-2736(94)00228-2

Besides secondary active transporters, primary active carriers translocate substrates across the membrane [14–16].

Three prototypes of primary active ATP-dependent transport systems are located at the canalicular membrane of liver cells:

(1) The multidrug resistance (mdr) gene product GP 170 (glycoprotein 170) is expressed not only in multidrug resistant tumor cells but also in normal tissue [17]. Using an MDR 1 cDNA probe, intermediate expression of the GP 170 kDa protein was shown in the liver [17]. Using a monoclonal antibody MRK 16, the mdr 1 gene product was found exclusively on the biliary canalicular front of hepatocytes [18]. Daunomycin, a typical substrate of the mdr 1 gene product, is taken up into canalicular inside-out vesicles isolated from rat liver [19].

(2) A bile acid transporter which transports taurocholate and other bile acids by an ATP-dependent mechanism which does not involve the MDR system [20–23].

(3) A non-bile acid organic anion transporter that transports among other things BSP, BSPGSH and leukotrienes [24–26]. This transport system is defective in TR^- rats which have a functional disorder resembling that seen in the Dubin-Johnson syndrome in man, sheep and monkies [24–27].

All of these transporters may be capable of transporting peptide drugs. The aim of this study, therefore, was to characterize the excretion of EMD 51921 into bile using isolated canalicular inside-out vesicles and to evaluate whether EMD 51291 is able to stimulate ATP-hydrolysis in canalicular plasma membranes. For the first time, we are able to provide evidence of an ATP-dependent transport mechanism for EMD 51921, and the existence of an EMD stimulatable ATPase in canalicular membranes of liver cells. Part of this work was presented at the XII International bile acid meeting in Basel on October 12–14, 1992 and appeared in abstract form [28].

2. Experimental procedures

2.1. Materials

The renin inhibitors EMD 51921 and [^{3}H]EMD 51921 (spec. act. 2.9 GBq/mmol) (Fig. 1) were gifts from Merck, Darmstadt, Germany. ATP, phenylmethylsulfonyl fluoride and sulfobromophthalein (BSP) were purchased from Serva, Heidelberg, Germany. ADP, AMP, GTP, CTP, UTP, creatine phosphate, creatine kinase, adenosine and vanadate were obtained from Boehringer, Mannheim, Germany. Filipin, vincristine sulfate, daunomycin hydrochloride were from Sigma, Deisenhofen, Germany. ECO plus 2000 was from Roth, Karlsruhe. Pyruvate kinase, phospho*enol*pyruvate, NADH, LDH, EGTA, imidazole and sodium azide were purchased from Merck, Darmstadt, Germany. [^{3}H]Taurocholate (spec. act. 77.7 GBq/mmol), [^{3}H]daunomycin (spec. act. 122.1 GBq/mmol),

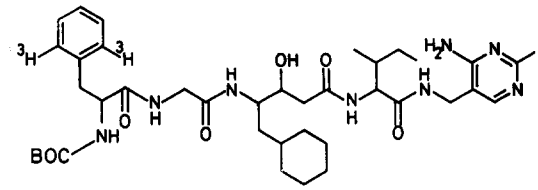

Fig. 1. Chemical structure of [^{3}H]EMD 51921.

[^{3}H]alanine (spec. act. 3145.0 GBq/mmol) were from NEN-Du Pont, Dreieich, Germany. All the other chemicals used were of analytical grade.

2.2. Animals

Male Wistar rats, weighing 280–320 g, maintained on a standard diet and water ad libitum, were used for most of the experiments. In experiments designed to evaluate the endogenous transporter, mutant Tr^- rats with a hepatobiliary transport defect in glutathione conjugates were used [26]. Tr^- rats were generously supported by Dr. Oude Elferink and Dr. P.L.M. Jansen (Academic medical Center, Amsterdam, The Netherlands).

2.3. Methods

Preparation of plasma membrane vesicles from rat liver

Rat liver canalicular and sinusoidal plasma membrane vesicles were isolated and characterized as described previously [29]. Canalicular inside-out vesicles were prepared by chromatography on Concanavalin A Sepharose [24]. The vesicles were stored in liquid nitrogen for up to 4 weeks before the experiments were performed. Before use, the frozen samples were quickly-thawed by immersion in a 37°C water bath. Protein concentration was determined with Bradford-reagent using bovine serum albumin as a standard [30].

Measurement of [^{3}H]EMD 51921, [^{3}H]taurocholate, [^{3}H]alanine and [^{3}H]daunomycin uptake by plasma membrane vesicles

The quality of vesicles prepared was estimated by measuring the uptake of alanine. Only preparations which showed uptake rates for alanine in NaSCN buffer in the range of 300–400 pmol/mg per min [31] were used. Unless otherwise noted, the incubation medium for transport contained plasma membrane vesicles (80–100 μg), varying concentrations of [^{3}H]EMD 51921, [^{3}H]taurocholate or [^{3}H]daunomycin, 250 mM sucrose, 10 mM Tris, 10 mM Mg_2Cl, with or without 2 mM ATP, 10 mM creatine phosphate, and 100 μg/ml creatine kinase (pH

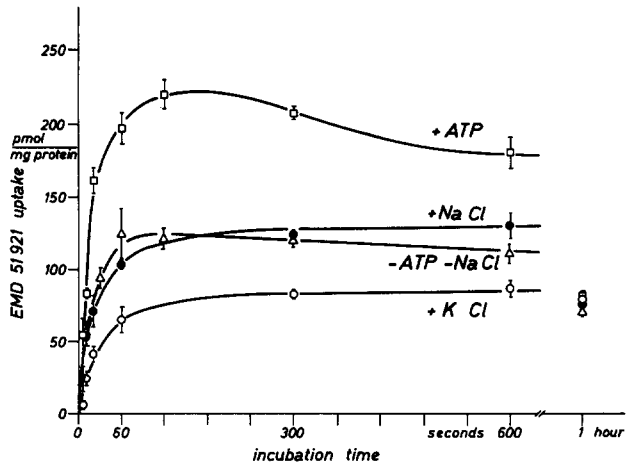

Fig. 2. [^{3}H]EMD 51921 uptake by canalicular inside-out vesicles (c-IV). [^{3}H]EMD 51921 uptake was measured in an incubation medium containing 3 μM [^{3}H]EMD 51921 into c-IV at 37°C in the presence or absence of an ATP regenerating system: 2 mM ATP, 10 mM creatine phosphate, 100 μg/ml creatine kinase, 10 mM Tris, 10 mM $MgCl_2$, 250 mM sucrose, 80–100 μg of protein, in a final volume of 125 μl. In the absence of ATP, uptake was measured in the presence of a sodium (100 mM NaCl, 150 mM sucrose, 10 mM Tris, 10 mM $MgCl_2$, pH 7.4, ●) or a potassium gradient (100 mM KCl, ○). Data represent means ± S.D. of triplicate determinations in three separate membrane preparations. ATP present, □; ATP absent, △.

7.4) in a final volume of 125 μl. After equilibration at 37°C, transport was initiated by adding 25 μl of vesicles to 100 μl of incubation buffer. After the indicated time periods, aliquots of the incubation mixture were used to determine substrate uptake. Transport was terminated by adding 1 ml ice-cold stop solution (100 mM sucrose, 0.2 mM $CaCl_2$, 100 mM KCl, 10 mM Tris-HCl, pH 7.4). Substrate incorporated into the vesicles was measured by rapid filtration using Millipore membrane filters (0.22 μm, type GVWP, Millipore, Bedford, MA) or nitrocellulose filters (0.22 μM, Sartorius, Göttingen, Germany). After washing the vesicles on the filters with 3 ml stop solution, radioactivity on the filters was measured in a liquid scintillation spectrometer (Raytest, PW 4700, Straubenhard, Germany).

To discriminate between transport of [^{3}H]EMD 51921 into an osmotic reactive intravesicular space and binding to the plasma membrane, vesicles were pretreated at 25°C for 60 min with various concentrations of sucrose. Transport was measured in the presence and absence of an ATP regenerating system. After 10 s and again after 30 s transport was terminated by addition of ice-cold stop solution adjusted to the osmolarity of the vesicle suspension.

All the experiments were repeated at least three times with different plasma membrane preparations. Values are expressed as mean ± S.D. after correction for radioactivity found on the filters in the absence of membrane vesicles. Kinetic evaluations were performed using the program BMDPAR on a Cyber 960 computer.

Assay of rat liver canalicular EMD stimulatable ATPase

Rat liver canalicular plasma membranes isolated according to the method of Meier et al. [29] were resuspended in 10 mM Tris-HCl (pH 7.4). ATPase activity was determined by a coupled spectrophotometric assay [32]. One unit is defined as the amount of enzyme that is capable of hydrolyzing 1 μmol of ATP per min at 37°C.

50 μl samples of canalicular membranes (200 μg of protein) were assayed for EMD stimulatable ATP hydrolysis. In a final volume of 1.11 ml, the assay medium contained: 1 mM EGTA, 1 mM ouabain, 20 mM NaN_3, 1.5 U/ml LDH, 3 U/ml pyruvate kinase, 625 μM NADH, 375 μM phospho*enol*pyruvate, 95 mM NaCl, 62.5 mM NH_4Cl, 3.125 mM $MgCl_2$, and 62.5 mM imidazole/HCl at pH 8.0. EMD was added at the concentrations indicated. The reaction was started by the addition of 50 μl of ATP (2 mM final concentration).

To investigate the effect of inhibitors of ATPases on the stimulation of ATP hydrolysis by EMD 51921, membranes were preincubated at 37°C with NEM (50 μM), oligomycin (9 μg/ml), 4-hydroxymercuribenzoate (50 μM), vanadate (25 μM) and ascorbate (5 mM) for 10 min before starting the ATPase assay.

3. Results

3.1. ATP-dependent transport of [^{3}H]EMD 51921 into canalicular inside-out plasma membrane vesicles

Uptake measurements into isolated canalicular inside-out plasma membrane vesicles by the filtration assay described [31], were only possible by using special hydrophilic filters (GVWP, Millipore, Bedford, MA) to reduce unspecific binding of the radioactively labeled substrates to the filter matrix. In this case, the filter blank ranged from 4–9% of [^{3}H]EMD 51921, 2–3% of [^{3}H]taurocholate and [^{3}H]daunomycin and 1–2% of

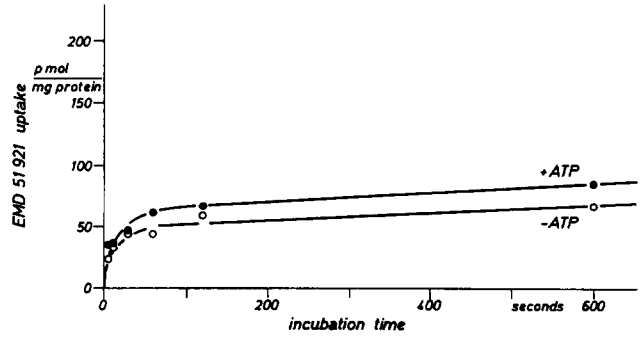

Fig. 3. Uptake of [^{3}H]EMD 61921 into basolateral plasma membrane vesicles isolated from rat liver. Uptake of the peptide into basolateral plasma membrane vesicles was measured in the presence and absence of ATP and an ATP regenerating system as described in Materials and methods. ATP present, ●; ATP absent, ○.

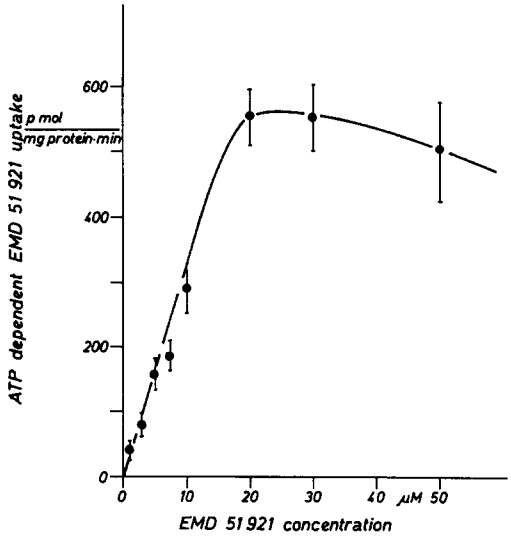

Fig. 4. Concentration dependence of ATP-dependent transport of [^{3}H]EMD 51921. The kinetics of uptake were measured in a medium containing various concentrations of [^{3}H]EMD 51921 (1–50 μM), 250 mM sucrose, 10 mM Tris, 10 mM $MgCl_2$, pH 7.4, in the presence and absence of 2 mM ATP, 10 mM creatine phosphate and 100 μg/ml creatine kinase at 37°C. ATP-dependent uptake was calculated as the difference of uptake in the presence and absence of ATP and an ATP regenerating system. Data represent triplicate determinations in three separate membrane preparations.

[^{3}H]alanine added. In the presence of 2 mM ATP (regenerating system), vesicle-associated [^{3}H]EMD 51921 rapidly increased at 37°C to a maximum value at 2–3 min, followed by a gradual decrease to equilibrium (Fig. 2). In the absence of ATP, a sodium gradient led to a higher uptake than a potassium gradient (Fig. 2). In basolateral plasma membrane vesicles the presence of ATP did not lead to a significant stimulation of the transport of [^{3}H]EMD 51921 (Fig. 3).

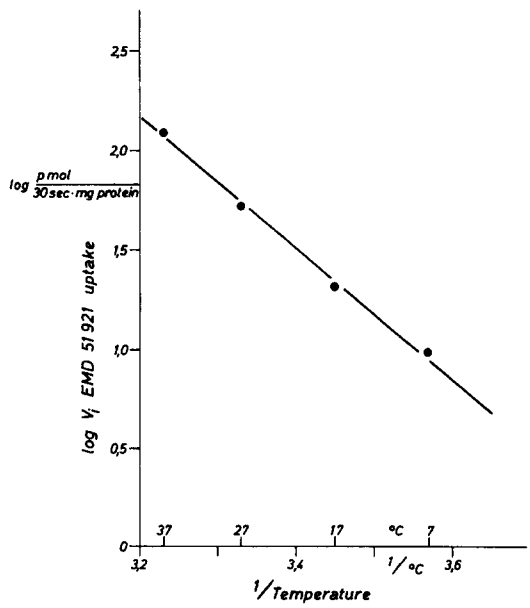

Fig. 5. Arrhenius-diagram of temperature-dependent uptake of [^{3}H]EMD 51921. EMD 51921 uptake was measured at 7, 17, 27, 37°C in an incubation medium containing 3 μM [^{3}H]EMD 51921 in the presence of ATP and an ATP regenerating system. An apparent activation energy of 62 kJ/mol was determined using the equation $A_{app} = 2.303 \times R \times \log V_i \times (1/T)^{-1}$.

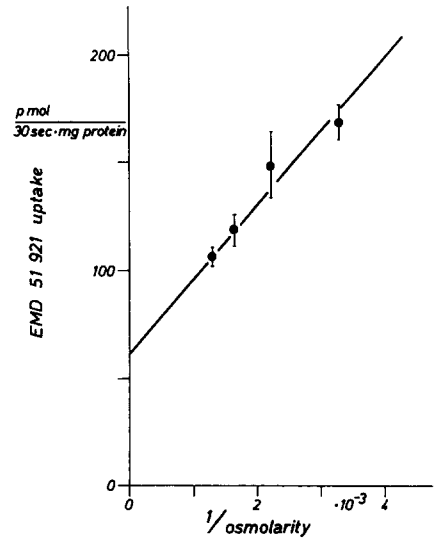

Fig. 6. Effect of medium osmolarity on [^{3}H]EMD 51921 uptake in c-IV. The ATP-dependent uptake of [^{3}H]EMD 51921 was measured in c-IV pretreated for 60 min at 25°C with 300–750 mM sucrose. EMD 51921 uptake was observed at 37°C. [^{3}H]EMD 51921 concentration was 3 μM. Uptake was terminated after 30 s. Data are presented as means ± S.D. ($n = 3$).

[^{3}H]EMD 51921 transport is concentration and temperature dependent. Kinetic evaluation revealed a saturable ATP-dependent transport with an apparent K_m of 12 μM and an apparent V_{max} value of 663 pmol/mg per min (Fig. 4). There was little increase in vesicle-associated [^{3}H]EMD 51921 uptake by ATP at 7°C. Increasing the temperature from 7°C to 17°C, 27°C and 37°C increased the ATP-dependent uptake of [^{3}H]EMD 51921 (7°C: 9 ± 1 pmol/mg per 30 s; 17°C: 20 ± 2; 27°C: 53 ± 3; 37°C: 123 ± 6 pmol/mg per 30 s). Q_{10} values were between 2.4–2.6. By transforming the data given above according to Arrhenius, an apparent activation energy of 62 kJ/mol was determined for 3.4 μM of [^{3}H]EMD 51921 at 37°C (Fig. 5).

ATP-dependent uptake occurred into an osmotic reactive intravesicular space. Vesicle-associated [^{3}H]EMD 51921 uptake decreased by increasing osmolarity of the medium (Fig. 6). After 30 s of uptake, 33% of the vesicle-associated radioactivity was due to binding.

ATP-dependent uptake of [^{3}H]EMD 51921 is influenced by the pH of the incubation medium. At pH 7.4 ATP-dependent uptake is lower (33 ± 0.7 pmol/mg per 10 s) compared to pH 8.0 (61 ± 4 pmol/mg per 10 s) and pH 6 (39 ± 6 pmol/mg per 10 s).

3.2. Effect of nucleotides on [^{3}H]EMD 51921 transport

[^{3}H]EMD 51921 transport was measured after replacing ATP with various nucleotides. AMP, GTP, CTP, UTP and

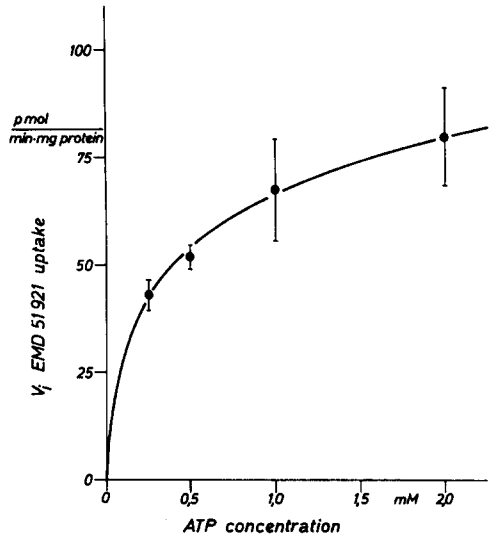

Fig. 7. Effect of ATP concentration on ATP-dependent uptake of [^{3}H]EMD 51921. Uptake of the peptide was measured in the presence of various concentrations of ATP and an ATP regenerating system.

AMP-PNP did not increase [^{3}H]EMD 51921 uptake. The same was also true for adenosine. In contrast, ATP and ADP increased [^{3}H]EMD 51921 uptake in the presence of an ATP regenerating system (creatine kinase and creatine phosphate) (Table 1). [^{3}H]EMD 51921 transport showed saturation kinetics with respect to ATP. An apparent Michaelis constant of 280 μM and an apparent maximum enzyme reaction velocity of 90 pmol/mg per 10 s were determined by computer analysis of the data (Fig. 7). ATP-dependent transport was inhibited after preincubation with vanadate, which acts as a transitional analogue for phosphate enzyme complexes [33] (Table 1). Ouabain, EGTA and NaN_3, however, had no effect (data not shown).

Table 1
Effect of nucleotides and preincubation with vanadate on the uptake of [^{3}H]EMD 51921

	[^{3}H]EMD 51921 uptake (pmol/mg per 30 s)
Minus ATP	74±4
Plus ATP	162±9
Adenosine	78±3
AMP	78±1
ADP	127±10
GTP	69±5
CTP	70±4
UTP	72±3
AMP-PNP	60±3
Plus vanadate 10 μM	68±7
100 μM	45±5

Uptake of the peptide was measured in the presence of ATP, ADP, AMP, adenosine, AMP-PNP, GTP, UTP and CTP (2 mM) in the presence of an ATP regenerating system at 37°C for 30 s. Data are presented as means ± S.D. (n = 3).

Table 2
Comparison of the uptake of [^{3}H]EMD 51921, [^{3}H]taurocholic acid and [^{3}H]daunomycin in canalicular plasma membrane vesicles isolated from normal and Tr$^-$ rats and mutual transport inhibition studies

	[^{3}H]EMD 51921 uptake (pmol/mg protein per 30 s)	Inhibition (%)
Normal	160±3	
Tr$^-$ rats	166±8	
Control	162±9	
Daunomycin		
10	113±7	30
100	63±8	60
Vincristine		
10	81±1	50
100	71±4	60
Taurocholate		
10	101±34	35
100	35±13	80
Filipin		
15	47±1	71
BSP		
10	105±20	35
100	40±8	75
DIDS		
50	162±7	
EGTA 1 mM	158±10	
Ouabain 1 mM	163±6	
NaN_3 1 mM	157±10	
	[^{3}H]Taurocholic acid uptake (pmol/mg protein per 30 s)	
Control	30±1	
EMD 51921		
10	13±0.51	54
100	7±0.3	76
	[^{3}H]Daunomycin uptake (pmol/mg per 30 s)	
Control	620±24	
EMD 51921		
10	624±13	
100	615±17	

Transport of [^{3}H]EMD 51921 was measured in canalicular plasma membrane vesicles isolated from normal and Tr$^-$ rats. The uptake of [^{3}H]EMD 51921, [^{3}H]taurocholic acid and [^{3}H]daunomycin was measured in the presence of either 10 or 100 μM of taurocholate, EMD 51921, BSP or daunomycin and vincristine. In the case of filipin the membranes were pretreated for 10 min with 15 μM of filipin. The incubation medium contained in a final volume of 125 μl: 3 μM of [^{3}H]EMD 51921, 1 μM of [^{3}H]taurocholic acid or 10 μM [^{3}H]daunomycin, vesicles (80–100 μg), 2 mM ATP and an ATP regenerating system, 10 mM Tris, 250 mM sucrose, 10 mM $MgCl_2$, pH 7.4. After 30 s of incubation, vesicle associated radioactivity was measured. Data represent the mean ± S.D. of triplicate determinations of three different plasma membrane preparations

3.3. Effect of taurocholate, vincristine and daunomycin on the ATP-dependent uptake of [^{3}H]EMD 51921

The ATP-dependent transport of [^{3}H]EMD 51921 is inhibited in the presence of taurocholate but also in the presence of vincristine and daunomycin, typical substrates of the P-glycoprotein [17–19]. [^{3}H]EMD 51921, however,

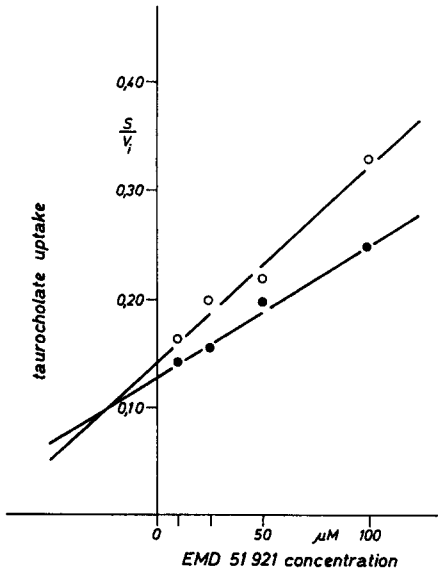

Fig. 8. Cornish-Bowden plot of the uptake inhibition of taurocholate by EMD 51921. The uptake of taurocholate (1 and 5 μM) was measured into c-IV in a medium as described in Fig. 2 in the presence of 10, 25, 50 and 100 μM of EMD 51921. After 30 s, uptake was stopped. The amount of taurocholate taken up (pmol/mg per 30 s) was determined and the data were transformed according to Cornish-Bowden.

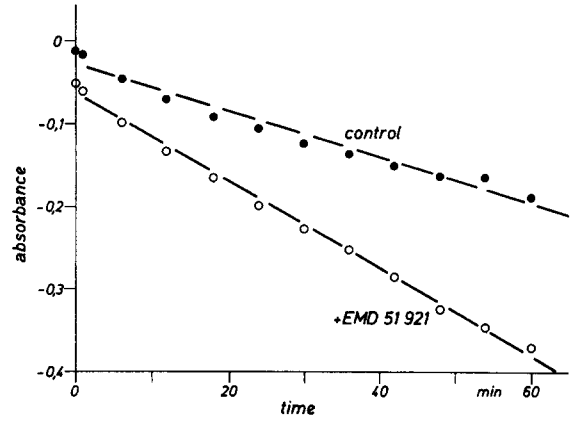

Fig. 9. Stimulation of ATP-hydrolysis by EMD 51921. Canalicular plasma membranes (200 μg/50 μl) were quickly thawed and added to 1 ml of buffer (at 37°C) as described under Materials and methods. The buffer contained 10 μl EMD 51921 (200 μM) or 10 μl DMSO (control). The reaction was started by addition of 50 μl ATP (2 mM) and was measured at 366 nm over a period of 60 min (interval time 12 s).

only inhibits the ATP-dependent uptake of taurocholate, whereas the ATP-dependent uptake of daunomycin is not reduced by EMD 51921 in equimolar concentrations (Table 2). Kinetic evaluations revealed that taurocholate and EMD 51921 are mutually un-competitive transport inhibitors (Fig. 8). Filipin, which inhibits taurocholate uptake but leaves daunomycin uptake unaltered [21], decreases ATP-dependent [^{3}H]EMD 51921 uptake. In Tr$^-$ rats lacking the canalicular ATP-dependent transport of cysteinyl leukotrienes and related anions, ATP-dependent transport of EMD 51921 was not altered (Table 1). BSP, however, a substrate of the multispecific organic anion transporter (MOAT), blocked [^{3}H]EMD 51921 uptake, whereas DIDS had no effect (Table 1).

3.4. Effect of EMD 51921 on ATP-hydrolysis in isolated canalicular plasma membranes

In the presence of ouabain, EGTA and NaN_3 in the buffer, addition of EMD 51921 resulted in a 1.8–2.5-fold increase of basal ATP-hydrolysis (0.021 U/mg protein) (Fig. 9). ATPase activity was stimulated by micromolar concentrations of EMD 51921. Analysis of a Lineweaver-Burk plot revealed an apparent K_m of 68 μM and a V_{max} of 0.033 U/mg protein (Table 3). The affinity for ATP was tested using increasing concentrations of ATP and 200 μM of EMD 51921 at pH 8.0. The apparent K_m value for ATP is 170 μM and the V_{max} is 0.075 U/mg protein (Table 3). The effect of pH on ATPase activity was tested with 200 μM of EMD 51921, 2 mM of ATP in the range of pH 8.5–8. ATP hydrolysis is increased at pH 8.0. ATP-hydrolysis in canalicular plasma membranes is not stimulated by alanine and by fragments of EMD 51921, which are not transported in an ATP dependent manner (data not shown). EMD 51921 does not stimulate ATPase activity of the Na^+/K^+-ATPase isolated from kidney cortex (data not shown). The EMD 51921 stimulatable ATPase cannot hydrolyze GTP (data not shown).

3.5. Effects of inhibitors of ATPases on EMD 51921 stimulatable ATPase in canalicular plasma membranes

The ATPase activity was characterized with respect to its inhibitor sensitivity. Various agents that are known to inhibit F, P and V type ATPases were tested [33]. The EMD-stimulatable ATPase is inhibited by preincubation of the membranes with vanadate, *N*-ethylmaleimide,

Table 3
(A) Kinetics of EMD stimulatable ATP-hydrolysis by canalicular plasma membranes of rat liver and (B) pH-dependence of EMD-stimulatable ATPase

(A)		
K_m for EMD	68 μM	
V_{max} for EMD	0.032 U/mg protein	U = 1 μM ATP/min
K_m for ATP	170 μM	
V_{max} for ATP	0.075 U/mg protein	
(B)	**U/mg protein**	
pH 6.5	0.130 ± 0.06	
pH 7.4	0.072 ± 0.018	
pH 8.0	0.159 ± 0.03	

(A) Canalicular plasma membranes (200 μg/50 μl) were incubated with various concentrations of EMD 51921 (10–200 μM; 2 mM ATP, pH 8.0) or were assayed with various concentrations of ATP (0.05–5 mM; 200 μM EMD 51921; pH 8.0). The ATP-hydrolysis was measured at 366 nm over a period of 60 min as described under Materials and methods.
(B) ATP-hydrolysis was measured in the pH range of 6.5 till 8.0 with 200 μM EMD 51921 and 2 mM ATP.

Table 4
Effect of ATPase inhibitors on the stimulation of ATP-hydrolysis by EMD 51921

		U/mg protein
Control		0.044
NEM	50 μM	0.00
4-OH-MB	50 μM	0.00
Vanadate	25 μM	0.00
Oligomycin	9 μg/ml	0.00
EGTA	1 mM	0.044
Ouabain	1 mM	0.044
NaN_3	20 nM	0.044

Canalicular plasma membranes (200 μg/50 μl) were treated with NEM (50 μM), vanadate (25 μM), 4-hydroxymercuribenzoate (50 μM) and oligomycin (9 μg/ml) at 37°C for 10 min. The pretreated canalicular plasma membranes were added to 1 ml of buffer (37°C) as described under Materials and methods. The buffer contained 200 μM EMD 51921 or 10 μl DMSO. The reaction was started by addition of 2 mM ATP and was measured at 366 nm over a period of 60 min.

oligomycin, 4-hydroxymercuribenzoate and ascorbate (Table 4). EGTA, ouabain and NaN_3 had no effect.

4. Discussion

4.1. ATP-dependent transport

In this study evidence is presented for the involvement of an ATP-dependent transport mechanism in the biliary secretion of the metabolically stable pseudopeptide EMD 51921. In contrast, in the sinusoidal domain of liver cells, such a primary active transporter is not operational.

In the presence of 2 mM ATP (regenerating system), uptake into the canalicular vesicles, but not into vesicles from the basolateral part of hepatocytes, is stimulated 2–3-times more than in the absence of ATP. Overshoot phenomena occur at 2–3 min of uptake, followed by a gradual decrease. Equilibrium is reached at 30–60 min depending on the amount of ATP added. ATP-dependent uptake follows saturation kinetics with respect to ATP concentration (K_m 280 μM) and EMD 51921 concentration (K_m 12 μM), indicating a carrier-mediated transport system. In addition, there is an ATP-independent uptake system for EMD 51291, which is more effective in the presence of a sodium gradient than in a potassium-gradient. The involvement of driving forces other than the presence of ATP for the uptake of EMD 51921 is under current investigation.

ATP-dependent transport of EMD 51921 depends on the pH of the incubation medium. A double-optimum pH is found with the highest uptake rates at pH 8. The same could also be shown for the EMD stimulatable ATPase activity. The reason of this phenomenon is uncertain at present.

Transmembrane transport of EMD 51921 is evident by the dependence of transport on temperature and osmolarity. Q_{10} values higher than 2 and an apparent activation energy of 62 kJ/mol indicate a protein mediated transport mechanism. Most of the peptide is taken up inside the vesicles. 33% of EMD 51921 is bound to the membrane after 30 s of uptake.

Transport specifically requires ATP, since in the presence of GTP, CTP and UTP uptake was not stimulated. Hydrolysis of ATP may be required for EMD uptake since transport is inhibited by vanadate, and the nonhydrolyzable ATP analog AMP-PNP failed to stimulate drug accumulation.

Since EMD 51921 is cationic (pK 5.8 and 8.9) under physiological conditions, we questioned whether MDR gene products are involved in biliary excretion of the peptide. In liver cells mdr gene products are exclusively expressed at the canalicular domain of hepatocytes [18,34] with highest levels of mdr2 and coexpression of mdr1a (mdr3) whereas mdr1b is missing [34]. The mdr1a/b gene products are believed to bind and transport various anticancer drugs and cationic bulky compounds with molecular weights of 400–1300, e.g., daunomycin and vincristine [35–37], and to interact with peptides such as *N*-acetyl-leucylleucylnorleucyl [38] and cyclosporin A [39]. In the canalicular plasma membrane of rat liver mdr1a (mdr3) is thought to be responsible for the transport of daunomycin [19,34]. Vincristine and daunomycin inhibit the ATP-dependent uptake of EMD 51921. EMD 51921, however, did not block ATP-dependent uptake of daunomycin. From these results it appears that the P-glycoprotein, responsible for the uptake of daunomycin in rat canalicular plasma membranes, is not involved in the elimination of EMD 51921. The inhibition of EMD 51921 uptake by the vinca alkaloid and anthracyclin may be due to binding of the compounds to the transporter without being transported themselves. This phenomenon was also found for progesterone, which efficiently inhibited azidopine binding to P-glycoprotein, but is not transported by P-glycoprotein [40]. A further hint supporting the fact that the daunomycin carrier is not involved in EMD 51921 uptake is yielded by studies with filipin. Filipin, an antifungal antibiotic which forms complexes with cholesterol, inhibits the uptake of EMD 51921 but which has no effect on the uptake of daunomycin and BSP [19]. At present, the involvement of other mdr gene products (mdr1a, mdr2 [41]) in the biliary elimination of EMD 51921 in the rat is uncertain.

Using Tr^- rats, which lack ATP-dependent multispecific organic anion uptake (MOAT), we were able to show that this transport system is not involved in EMD 51921 excretion, although BSP, a substrate of this transporter [25], inhibited the uptake of EMD 51921. Inhibition of ATP-dependent transport by BSP was also shown for taurocholate [21]. Since BSP uptake is not influenced by filipin [19] whereas EMD 51921 uptake is, and since uptake of EMD 51921 is unaltered in TR^- rats, it is suggested that the MOAT is not involved in EMD 51921 uptake. This is further substantiated by the fact that EMD

51921 is positively charged under physiological conditions and that the MOAT preferentially transports dianionic compounds [24–27].

Mutual transport inhibition was found for taurocholate and EMD 51921, the type of inhibition, however, turned out to be non- or uncompetitive. In addition, these transport systems differ in their response to certain transport inhibitors. The ATP-dependent taurocholate transporter is not inhibited by daunomycin [21], whereas the EMD transporter is. Vanadate at 100–200 μM inhibits both taurocholate and EMD 51921 uptake, DIDS on the other hand has no effect on the uptake of EMD 51921.

4.2. EMD 51921 stimulatable ATP-hydrolysis

In addition to ATP-dependent transport of EMD 51921, a complementary activity of ATP hydrolysis that is dependent upon the presence of EMD 51921 could be demonstrated. A basal ATPase activity could be measured in canalicular plasma membranes, which did not result from the activity of the Na^+/K^+-ATPase or mitochondrial ATPases as contaminants of our membrane preparation, since ouabain, NaN_3 and EGTA, known inhibitors of these ATPases, were included in the buffers. Adding EMD 51921 resulted in a 1.8–2.5-fold increase in ATPase activity, which could be measured with a coupled spectrophotometric assay. The process shows saturation kinetics with respect to EMD 51921 and ATP concentration and is pH dependent as was also shown for the transport activity. The K_m values for EMD 51921 in the transport and the enzyme assays are in the micromolar range (12 versus 68 μM), as are the K_m values for ATP (280 versus 170 μM). Several transport ATPases have been previously characterized, however, inhibitor profiles suggest that EMD-stimulatable ATPase may be different. The ATPase activity as well as the transport of the peptide are affected by vanadate, a potent inhibitor of P-type ATPases (e.g., Na^+/K^+-ATPase). NEM, which blocks P-type ATPases but also vacuolar ATPases [42] as well as oligomycin, a blocker of mitochondrial ATPases [43], inhibit EMD stimulatable ATPase. Since NaN_3, inhibiting the enzymatic activity of F-type ATPases [42], has no inhibitory effect on EMD-stimulatable ATPase, the effect of oligomycin is not due to mitochondrial contamination of our plasma membrane preparation. Ouabain, the well known blocker of the Na^+/K^+-ATPase and EGTA had no affect on EMD-stimulatable ATPase. In addition, EMD 51921 does not influence the activity of the Na^+/K^+-ATPase, isolated from kidney cortex. Furthermore, stimulation of ATPase activity was not observed with the amino acid alanine or fragments of the peptide, which are not transported in an ATP-dependent manner.

In rat and human canalicular membranes, a glutathione *S*-conjugate dependent ATPase has been described that uses the free energy of ATP hydrolysis for transport of glutathione *S*-conjugates across the membrane [44–49]. This export pump [48] may be involved in the transport of GSH conjugates, leukotrienes and other organic anions [50], and may be identical to the MOAT system [24–26]. It does seem not to be related to the EMD-stimulatable ATPase since the former is stimulated by preincubation with thiol-depleting compounds such as NEM and hydroxymercuribenzoate [45], and the latter is not. This is in agreement with the transport studies, in which EMD 51921 was taken up by glutathione-*S*-conjugate transport-deficient Tr^- rats.

ATPase activity associated with P-glycoprotein and drug-stimulatable ATP-hydrolysis of human and hamster ovary cell MDR pumps has been reported [51,52]. In rat canalicular membranes, however, only ATP-dependent daunomycin transport has been measured [19]. No reports exist on the stimulation of ATPase activity by vinca alkaloids or anthracyclines in rat canalicular membranes. Comparing the influence of inhibitors on the activity of the human MDR 1 protein and the EMD-stimulatable ATPase, NEM is the compound with which both ATPases can be distinguished. ATPase activity of partially purified P-glycoprotein from Chinese hamster ovary cells, however [52] was inhibited by NEM and other sulfhydryl modifying reagents, as was EMD-stimulatable ATPase. Furthermore, it has been postulated that one of the physiological functions of P-glycoprotein is the outward transport of endogenous peptides [38]. In light of the results of the transport studies, we currently suggest that the mdr gene product responsible for the transport of daunomycin in rat canalicular membranes is not identical to the EMD-stimulatable ATPase. Further studies are needed to elucidate the involvement of mdr gene products in the biliary elimination of peptides in the rat.

The identity and mechanism of the canalicular bile acid transporter is currently a contentious issue in the liver field. Recently, a 100 kDa rat liver canalicular ecto-ATPase has been shown to function as canalicular bile acid transport protein after transfection of the cDNA for the ecto-ATPase in COS cells [53,54]. Transport of taurocholate into transfected COS cells, as well as the ecto-ATPase activity, are inhibited by DIDS [53]. This inhibition was not found for the EMD 51921 stimulatable ATPase. Neither transport of EMD 51921 nor ATPase activity were influenced by DIDS. Vanadate, which inhibits EMD 51921 transport and ATPase activity, only marginally blocks the ecto-ATPase activity [53]. We therefore suggest, that the EMD-stimulatable ATPase is distinct from the ecto-ATPase. The hypothesis that it is identical to the ATP-dependent taurocholate transporter has been controversial.

4.3. Conclusions

The peptide drug EMD 51921 is excreted into bile by an ATP-dependent mechanism. The ATP-dependent transport protein has ATPase activity, which is stimulated in the presence of EMD 51921. Our results suggest that the EMD

stimulatable ATPase is not related to an organic anion export pump [24–26] or the daunomycin transporting mdr gene product in rat liver canalicular plasma membranes. Although EMD 51921 and taurocholate are mutual transport inhibitors, the non- or uncompetitive type of inhibition points to the distinctness of the two transport systems. Further studies are needed to identify and isolate the EMD 51921 pseudopeptide transporter.

Acknowledgements

We are indebted to Dr. B. Boschek for critically reading the manuscript. Dr. Peter L.M. Jansen and Dr. R. Oude Elferink kindly provided the transport mutant rats. We gratefully acknowledge Dr. Schmitges, Dr. Raddatz and Dr. Faro for supplying us with the renin-inhibitors. This work was supported by grants from the Deutsche Forschungsgemeinschaft SFB 249.

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