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First-Pass Elimination of a Peptidomimetic Thrombin Inhibitor is Due to Carrier-Mediated Uptake by the Liver

INTERACTION WITH BILE ACID TRANSPORT SYSTEMS

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ABSTRACT. CRC 220 (4-methoxy-2, 3, 6-trimethylphenylsulfonyl-L-aspartyl-D-4-amidinophenylalanyl-piperidide) is a competitive peptide-based trombin inhibitor with high affinity to human α-thrombin (K_i 2.5 nM). The amphiphilic compound exhibits virtually no systemic bioavailability despite proteolytic stability and proven enteral absorption. After intravenous application (V. jejunalis) in rats CRC 220 is almost completely excreted into bile. Simultaneous administration of bile acids considerably decreases this first-pass elimination. CRC 220 is extensively taken up in isolated rat hepatocytes by a saturable carrier-mediated transport with K_m 23.7 μM and V_{max} 775 pmol × mg^{-1} × min^{-1}. A large part of this transport is energy-dependent. At temperatures above 20°C, the uptake is accelerated exponentially. The activation energy amounts to 82 kJ/mol. A minor portion of CRC 220 uptake occurs by physical diffusion with a permeability coefficient of 7.83 × 10^{-7} cm/sec at 12°C. Sodium ions energize CRC 220 uptake. Replacement of sodium by choline or lithium decreases the transport rate of 23–40%. In addition, a negative membrane potential facilitates the uptake. CRC 220 transport is only observed in hepatocytes: it is absent in BHK, FAO, HepG2, HPCT 1E3, and HPCT 1E3-TC cells. In the presence of 4-amidinophenylalanine derivatives, CRC 220 uptake is considerably decreased. Inhibition also occurs with bile acids and bromosulfophthalein, but less with bumetanide. Because CRC 220 inhibits bile acid uptake into hepatocytes and *vice versa*, the results suggest that the first-pass elimination of this amphiphilic thrombin inhibitor is due to an active carrier-mediated transport process in the basolateral plasma membrane of rat hepatocytes, and that this transport occurs *via* a bile acid transport system. BIOCHEM PHARMACOL 52;1:85–96, 1996.

KEY WORDS. blood coagulation; CRC 220; bile elimination; membrane transport; bioavailability; hepatocyte

The importance of biologically active peptide drugs has increased in modern pharmacology [1]. Peptide antibiotics, modified peptide hormones and peptide enzyme inhibitors have been developed and introduced for clinical purposes [2–10]. To envisage an oral application, the peptide derivatives should pass the gastrointestinal tract and reach the systemic blood circulation without degradation. Very often, however, peptide drugs are excreted by the liver [11]. This first-pass elimination reduces drug bioavailability and imperils clinical practicability.

CRC 220§ is a modified amphiphilic dipeptide that competitively inhibits thrombin with a K_i value of 2.5 nM [12]. It was developed to inhibit blood coagulation transiently [13]. CRC 220 is absorbed from the gut without degradation [13, 14]. However, the dipeptide derivative exhibits virtually no systemic bioavailability. Low bioavailability is a potential drawback of almost all synthetic peptide-based thrombin inhibitors [15]. They all show a very short half-life after intravenous, and especially after oral, application due to almost complete elimination into bile [16, 17]. To demonstrate small anticoagulant or antithrombotic effects, extremely high oral doses are often required [18, 19].

We have now analyzed the first step in the biliary elimination of CRC 220, namely permeation through the sinusoidal membrane of hepatocytes. Previous studies have shown that certain cyclic and linear hydrophobic peptides, such as cyclosomatostatins [20], some linear renin-

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§ *Abbreviations:* CRC 220, 4-methoxy-2,3,6-trimethylphenylsulfonyl-L-aspartyl-D-4-amidinophenylalanyl-piperidide; CRC 294, phenylmethylsulfonyl-L-aspartyl-4-amidinophenylalanyl-piperidide; CRC 369, 4-methoxy-2,3,6-trimethylphenylsulfonyl-aspartyl-phenylalanyl-piperidide; CRC 569, 4-methoxy-2,3,6-trimethylphenylsulfonyl-L-aspartyl-D-4-carbamoylphenylalanyl-piperidide; CRC 572, 4-methoxy-2,3,6-trimethylphenylsulfonyl-L-aspartyl-L-4-amidinophenylalanyl-piperidide; CRC 584, 4-methoxy-2,3,6-trimethylphenylsulfonyl-D-aspartyl-L-4-amidinophenylalanyl-piperidide; NAPAP, Nα-(2-naphthylsulfonyl-glycyl)-4-amidinophenylalanyl-piperidide; aspartame, L-aspartyl-L-phenylalanine methyl ester; CCCP, carbonylcyano-chlorophenylhydrazone; BSP, bromosulfophthalein.

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inhibiting peptides [21], and phallotoxins [22], enter liver cells by energy-dependent carrier-mediated transport. In competition experiments, it was found that the above peptides share a common transport system with the physiological bile acids [11]. This would elegantly explain the liver-specific clearance of these peptide drugs, which mimics hepatobiliary elimination of bile acids perfectly [23].

The aim of the present study is to characterize CRC 220 uptake in isolated rat hepatocytes and to determine structural elements relevant for its first-pass elimination by the liver.

MATERIALS AND METHODS

Materials

Thrombin inhibitors CRC 220/[^{14}C]CRC 220 (spec. act. 3275 MBq/g, 2090 MBq/mmol), CRC 294, CRC 369, CRC 569, CRC 572 and CRC 584 were generous gifts from Behringwerke AG (Marburg, Germany) (Fig. 1). NAPAP was a gift from Dr. J. Stürzebecher, Friedrich-Schiller-University Jena (Erfurt, Germany). [^{3}H]Cholic acid (spec. act. 0.93 TBq/mmol) and [^{3}H]taurocholic acid (spec. act. 96.2 GBq/mmol) were obtained from Du Pont-De Nemours (Dreieich, Germany). Collagenase was purchased from Boehringer-Mannheim (Mannheim, Germany) and the Bio-Rad Protein Assay from Bio-Rad Laboratories (Munich, Germany). Cell culture media, such as Dulbecco's modified Eagle medium (DMEM), Ham's F12, NCTC 135 medium, fetal calf serum, trypsin-EDTA, penicillin/streptomycin solution, and glutamine solution were from Flow (Meckenheim, Germany) or Gibco (Eggenstein, Germany). Insulin, dexamethasone, and inosin were obtained from Serva (Heidelberg, Germany) and 260 mL culture flasks from Nunc (Wiesbaden, Germany). All other chemicals and reagents were used in the highest purity available commercially.

Buffer Composition

Tyrode buffer (Na^+-Tyrode) in mM: 137 NaCl, 2.7 KCl, 1.05 $MgCl_2$, 1.8 $CaCl_2$, 12 $NaHCO_3$, 0.42 NaH_2PO_4, and 5.55 glucose, pH was adjusted to 7.4 at 37°C by 1 N HCl. Lithium-Tyrode buffer: NaCl was replaced by 137 mM LiCl and $NaHCO_3$ by 12 mM Li_2CO_3 (pH 7.4 at 37°C). Choline-Tyrode buffer: NaCl was replaced by 137 mM choline chloride and $NaHCO_3$ by 12 mM choline bicarbonate (pH 7.4 at 37°C). Nitrate-, thiocyanate-, and sulfate-Tyrode buffer: in these buffers, NaCl was replaced by either 137 mM $NaNO_3$, 137 mM NaSCN, or 137 mM Na_2SO_4. $CaCl_2$ was omitted after verifying its ineffectiveness.

In-situ *Bile Excretion Experiments*

Male Wistar rats (body weight 220–280 g) were anaesthetized by intraperitoneal injection of 1.7 mL of a 20% urethane solution. During the whole experiment, the animals were transferred to a box with temperature control and kept at 25°C to maintain the physiological body temperature. After inserting a small catheter into the common bile duct and fixing it with tissue adhesive, [^{14}C]CRC 220/CRC 220 (4.4 nmol/45.6 nmol) or a mixture of [^{14}C]CRC 220/CRC 220 and the unlabelled bile acids cholate (500 nmol or 2.5 μmol) or taurocholate (500 nmol) were applied *via* a jejunal vein by bolus injection. The radiolabelled compound was dissolved in 5 μL ethanol and subsequently diluted with 495 μL Tyrode buffer; the bile acids were also dissolved in this buffer. The final ethanol concentration was no more than 1%, v/v. After injection of the substances, bile was collected every min up to 10 min, every 5 min up to 60 min and, subsequently, every 10 min up to 2 hr. The bile-associated radioactivity of each fraction was determined in a liquid scintillation counter.

Preparation of Isolated Rat Liver Parenchymal Cells

Hepatocytes were prepared from male Wistar rats (body weight 220–280 g) by recirculating collagenase perfusion according to a modified protocol of Berry and Friend [24, 25]. Freshly isolated liver cells were used after an equilibration period of 30 min in Tyrode buffer, pH 7.4, at 37°C under carbogen atmosphere (95% O_2/5% CO_2) within 2 hr in a concentration of 2×10^6 hepatocytes/mL (3.8 mg cell protein/mL). The uptake studies were performed with preparations of 85–95% intact cells referring to the exclusion of 0.2% Trypan Blue. Protein concentrations were determined using the Bio-Rad reagent according to the method of Bradford [26].

Uptake Experiments

Standard uptake studies were performed with 2 mL hepatocyte suspensions (2×10^6 cells/mL) incubated in small glass flasks and shaken in a 37°C water bath under carbogen atmosphere. At 15, 45, 75, 105, 135 sec and 3, 5, and 10 min after addition of 0.89 μM [^{14}C]CRC 220, 40 nM/10 μM [^{3}H] taurocholic acid/taurocholate or 40 nM/10 μM [^{3}H] cholic acid/cholate, aliquots of 100 μL cell suspension were centrifuged across a silicon oil layer [27]. The cell pellet was solubilized in KOH overnight and cell-associated radioactivity was measured in a scintillation counter.

The initial uptake rate (V_i) was calculated in pmol × mg cell protein^{-1} × min^{-1} from the first 105 sec of uptake. In the kinetic studies, the initial uptake rates were plotted vs the substrate concentration according to Michaelis-Menten.

Temperature Dependency

The temperature dependency of the CRC 220 uptake was investigated in Tyrode buffer at 2, 12, 22, 32, and 37°C. Before uptake measurements, the cell suspension was incubated at the desired temperature for 5 min. The difference of V_i at each Δ10°C-interval gave the Q_{10}-values. The activation energy E (kJ/mol) was calculated using an Arrhe-

nius diagram from the slope of the graph log V_{max} versus $1/T$. The uptake curves at 12°C were used to determine the permeability coefficient P (cm/sec) of the energy-independent portion of CRC 220 uptake [28].

Energy Dependency

The energy dependency of CRC 220 uptake was analyzed with hepatocyte suspensions preincubated for 5 min in the presence of the metabolic inhibitors antimycin A (30 μg/mL) and oligomycin (10 μg/mL) and the protonophore carbonylcyano-chlorophenylhydrazone, CCCP (10 μg/mL). For controls, the appropriate volume of the solvent (ethanol; 10 μL/mL cell suspension) was added.

Uptake Experiments in Buffers Different from Na^+-Tyrode

After the equilibration period of 30 min in Na^+-Tyrode buffer, hepatocytes were washed 3 times with the appropriate buffers by short-term centrifugation for 2–3 sec. The final pellets were resuspended in the respective buffers 5 min prior to the uptake measurements.

Uptake Experiments in the Presence of Inhibitors

The liver cell suspensions were preincubated for 30 sec with various concentrations of CRC 220 substrate analogues, bile acids, BSP, and bumetanide. These potential inhibitors were dissolved either in the respective buffers or in 10 μL ethanol. Controls were performed with an appropriate volume of the buffer or ethanol. After the addition of the radiolabelled compound, transport studies were performed as described above.

The IC_{50} values were calculated from the graphs of the percentage uptake inhibition vs log of inhibitor concentration.

Binding Experiments with Permeable Cells

Cell binding of CRC 220 was determined with permeabilized hepatocytes. After the equilibration period, hepatocytes were frozen in liquid nitrogen and subsequently thawed at room temperature. By means of this procedure, cells become permeabile for Trypan Blue. These hepatocyte cell ghosts were used for binding experiments at 37°C as described above.

Intracellular Accumulation Ratio

To calculate the intracellular accumulation of CRC 220 in hepatocytes, the volume of intracellular water was taken to be 3.35 μL/10^6 hepatocytes [29, 30]. Calculation of the accumulated ratio took into account the maximum radiolabelled [^{14}C]CRC 220 uptake per mg cell protein; 1 mg of protein corresponds to 500,000 cells.

Cell Culture Conditions

The cell specificity of hepatic CRC 220 uptake has been proven with different hepatoma cell lines and baby hamster kidney cells. The hepatoma cells were human HepG2 cells [31], rat FAO Reuber hepatoma cells H35 [32, 33], and rat hepatocyte/hepatoma hybrid cells HPCT 1E3 [34]. The culture conditions were as follows: a) HepG2, DMEM medium, 10% fetal calf serum, 2 mM glutamine, penicillin/streptomycin (50 U/50 μg/mL); b) FAO cells, Ham's F12/NCTC medium (1:1, v/v), 5% fetal calf serum, 2 mM glutamine, penicillin/streptomycin (see above). The culture conditions for HPCT cells and BHK cells [35] were identical and are described elsewhere [34, 36]. All cell cultures were maintained in a humidified incubator at 37°C and 5% CO_2/air atmosphere. The cells were routinely cultured in 260-mL plastic culture flasks and used for transport studies when ⅔ confluency was reached.

Transport Studies

For the uptake measurements with 0.89 μM [^{14}C]CRC 220, 10.89 μM [^{14}C]CRC 220/CRC 220, 40 nM/10 μM [^{3}H] taurocholic acid/taurocholate and 40 nM/10 μM [^{3}H]cholic acid/cholate, the cultured cell lines were removed with trypsin-EDTA from the culture flasks and incubated as a single cell suspension in standard culture medium for 20 min. During this incubation period, the cells were gently shaken in small Erlenmeyer flasks under O_2/CO_2 atmosphere at 37°C. After 20 min, the culture medium was replaced by Tyrode buffer. After another incubation period of 10 min, uptake studies were performed with 1 mL cell suspension (6×10^6 cells/mL), as described above.

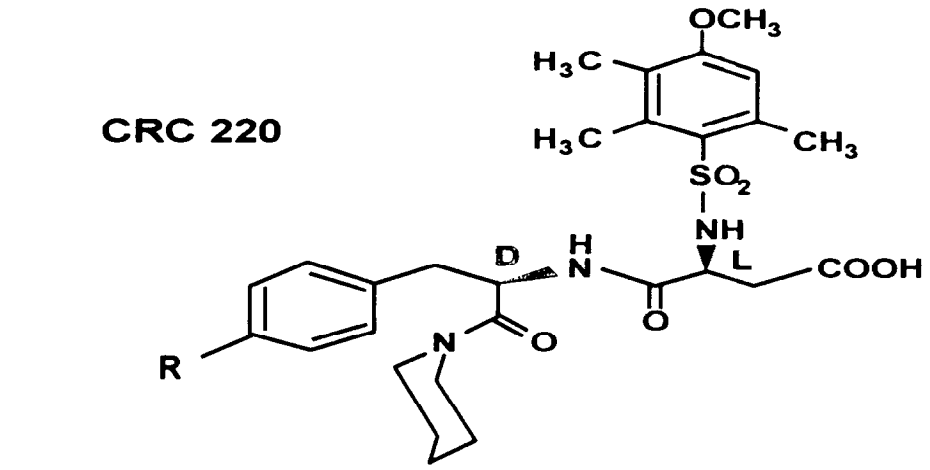
Statistical Methods

All experiments were performed several times with n different cell preparations. The results are expressed as mean ± standard deviation (SD). The Lineweaver-Burk diagram was calculated by a linear regression analysis; the IC_{50} values were determined by a logarithmic regression analysis. Significance was proven by variance analysis with the BMDP 2V program on a Cyber 860 computer.

RESULTS

Excretion of CRC 220 into Bile

CRC 220 was very rapidly excreted into bile. Maximum excretion was reached 5–7 min after intravenous (V. jejunalis) application. At that time, 50% of the ^{14}C-labelled compound was detected in bile. This value increased to 68 ± 7% after 10 min, to 88 ± 2% after 30 min and to 92 ± 1% after 2 hr (Fig. 2). Simultaneous administration of the physiological bile acids cholate and taurocholate significantly decreased the biliary excretion of the labelled thrombin inhibitor (Fig. 2), indicating a competition between bile acid and CRC 220 clearance by the liver.



	R	D	L
CRC 220	$C(NH)NH_2$	D	L
CRC 572	$C(NH)NH_2$	L	L
CRC 569	$CONH_2$	D	L
CRC 584	$C(NH)NH_2$	L	D
CRC 369	H	D	L

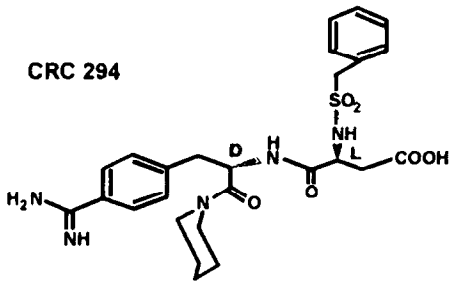


FIG. 1. Chemical structures of the thrombin inhibitors CRC 220, CRC 572, CRC 569, CRC 584, CRC 369, and CRC 294.

Time-course of CRC 220 Uptake in Isolated Rat Hepatocytes

The uptake of the thrombin inhibitor CRC 220 into isolated rat hepatocytes was time-dependent. The uptake rate was linear within the first 105 sec (Fig. 3a). At low (0.89 μM) and high (100.89 μM) concentrations, maximum uptake was reached after 20 min and, at middle (10.89 μM) concentrations, after 15 min (Fig. 3b). CRC 220 transport was biphasic: uptake was followed by a slow decline in cell-associated radioactivity over a period of 1 hr. This decrease was not caused by any cell membrane permeabilization due to toxic effects (analyzed by Trypan Blue), nor did it represent an efflux of CRC 220 metabolites. It appears that CRC 220 itself was pumped out of the cells upon long-time exposure. The intracellular accumulation was determined at the maximum of each uptake curve: it was 125-fold for 0.89 μM CRC 220, 62-fold for 10.89 μM, and 25-fold for 100.89 μM.

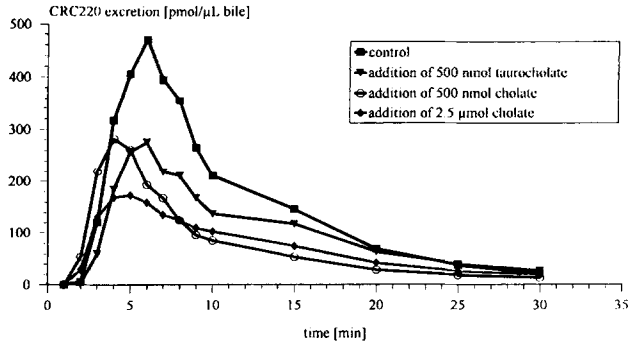


FIG. 2. Inhibition of biliary CRC 220 excretion by the bile acids taurocholate and cholate. After i.v. bolus application of 4.4/45.6 nmol [^{14}C]CRC 220/CRC 220 without (control) or with the indicated bile acids, bile was collected until 30 min at the times described in Materials and Methods. n = 3; mean ± SD; SD ≤ 25%.

Concentration Dependency of CRC 220 Uptake

When the concentration of the thrombin inhibitor was increased, the initial transport velocity (V_i) tended to reach a V_{max} (Fig. 4a). CRC 220 uptake in isolated rat hepatocytes followed mixed-type kinetics, which consisted of a large saturable transport component and a small nonsaturable uptake. The saturable portion of the transport was obtained by subtracting the values of the nonsaturable portion at 12°C, which was taken as diffusion, from uptake at 37°C, when both processes occurred simultaneously (Fig. 4a). The kinetic parameters for the saturable portion were obtained by a Michaelis-Mentin plot. The calculated K_m was 23.7 μM and the V_{max} 775 pmol/mg protein × min. Comparable results were obtained by Hanes-, Woolf-Hofstee, and Lineweaver-Burk plots (Fig. 4b).

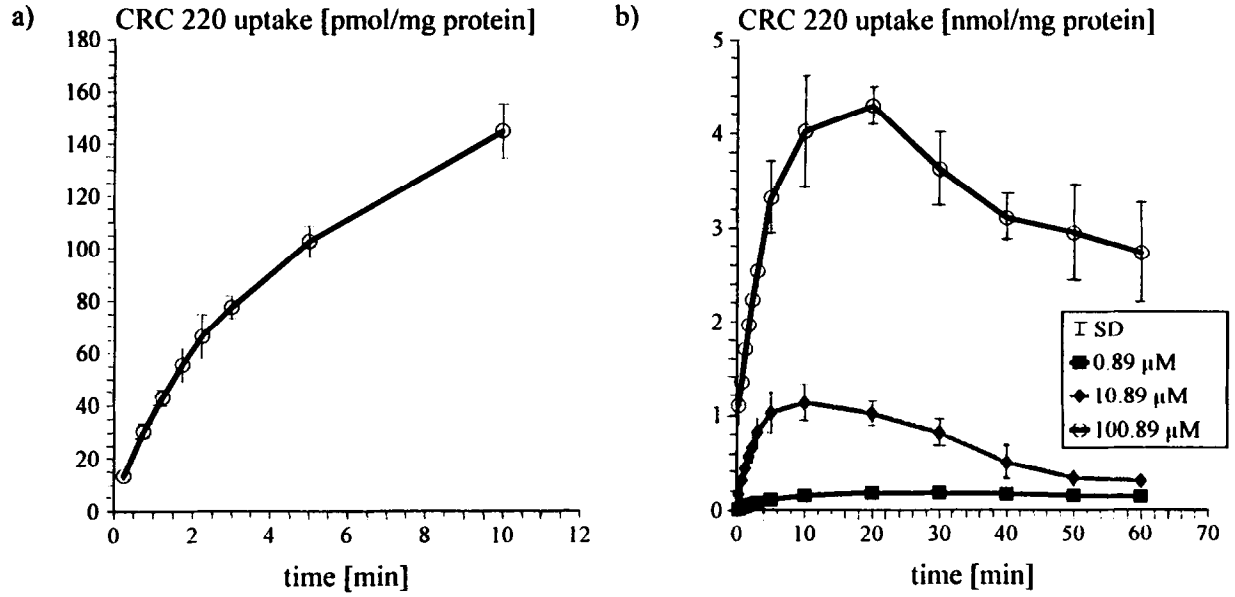


FIG. 3. Time and concentration dependency of the uptake of CRC 220 into isolated rat liver cells. (a) 2 mL of liver cell suspensions were incubated with 0.89 µM [^{14}C]CRC 220 and uptake was measured at 15 sec, 45 sec, 75 sec, 105 sec, 135 sec, 3 min, 5 min and 10 min. Initial velocity was calculated between 15 and 105 sec and was 27.8 ± 3.6 pmol/mg × min. r = 0.997; n = 4; mean ± SD. (b) Aliquots of 2 mL hepatocyte suspension (2 × 10^6 cells/mL) were incubated at 37°C with the indicated concentrations of CRC 220. Uptake was measured at the times described in Materials and Methods. n = 3; mean ± SD.

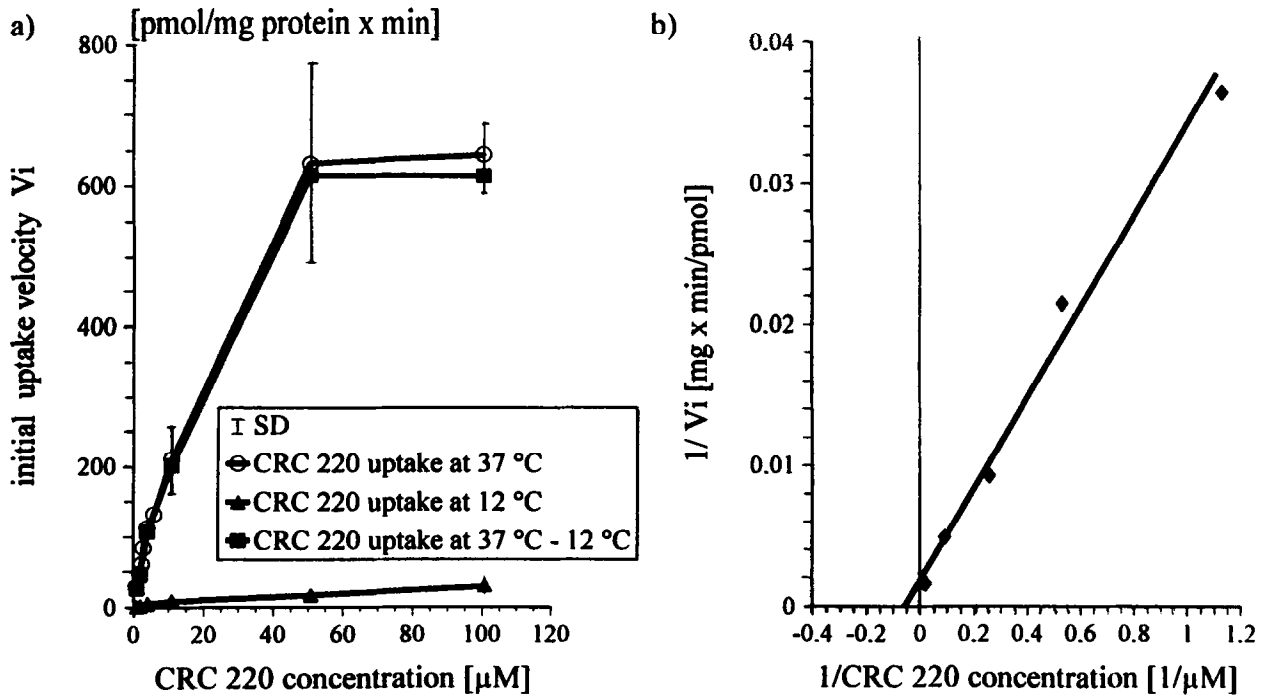


FIG. 4. Kinetics of CRC 220 uptake at 37°C and 12°C and determination of the saturable part of the transport. (a) Aliquots of 2 mL hepatocyte suspension were incubated with 0.89 µM [^{14}C]CRC 220 and different concentrations of unlabelled CRC 220. Initial velocity was calculated between 15 and 105 sec. Saturable transport was determined by subtracting the 12°C values from the 37°C curve. The permeability coefficient of P = 7.83 × 10^{-7} cm/sec was calculated from the slope of the diffusion curve (12°C) by linear regression analysis. n = 3; mean ± SD. (b) Lineweaver-Burk plot of the saturable uptake of CRC 220 in rat hepatocytes. r = 0.994; n = 3.

Effect of Temperature on CRC 220 Uptake, Calculation of the Activation Energies, and the Permeability Coefficient

The rapid uptake of the modified dipeptide was significantly decreased at low temperatures. At temperatures above 22°C, the initial uptake velocity increased exponentially (Fig. 5a). The Q_{10} value represents the increase in the uptake rate at a temperature interval of 10°C. Q_{10}-values exceeding 1.3 indicate an active transport process [37]. In experiments with CRC 220, this value rose from 1.6 between 2°C and 12°C to 3.6 between 22°C and 32°C.

The activation energy for the saturable portion of CRC 220 uptake, calculated by Arrhenius diagram, amounted to 82 kJ/mol (Fig. 5b); the permeability coefficient of the passive diffusion amounted to 7.83×10^{-7} cm/sec.

Energy Dependency of Hepatocellular CRC 220 Uptake

The energy dependency of hepatocellular uptake was investigated by preincubation of hepatocytes with the metabolic inhibitors CCCP, antimycin A, and oligomycin. All three inhibitors block energy utilization in the respiratory chain of the liver cell and are known to reduce hepatocellular ATP by more than 95% [22, 38]. The three compounds markedly inhibited the CRC 220 transport (Fig. 6).

Effect of Sodium Ion Substitution on Hepatocellular CRC 220 Uptake

When sodium was replaced by choline and lithium in Tyrode buffer, the uptake with two different CRC 220 con-

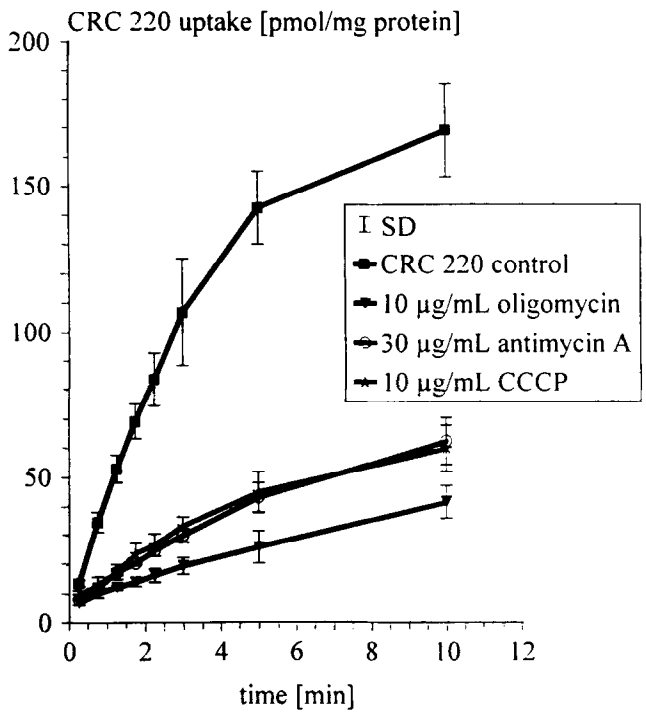


FIG. 6. Uptake of CRC 220 in the presence of metabolic inhibitors. Aliquots of 2 mL hepatocyte suspension were incubated with the indicated concentrations of oligomycin, antimycin A, and CCCP for 10 min prior to the addition of 0.89 µM [^{14}C]CRC 220. Initial uptake velocity was calculated between 15 and 105 sec. $P \leq 0.0001$. $n = 3$; mean ± SD.

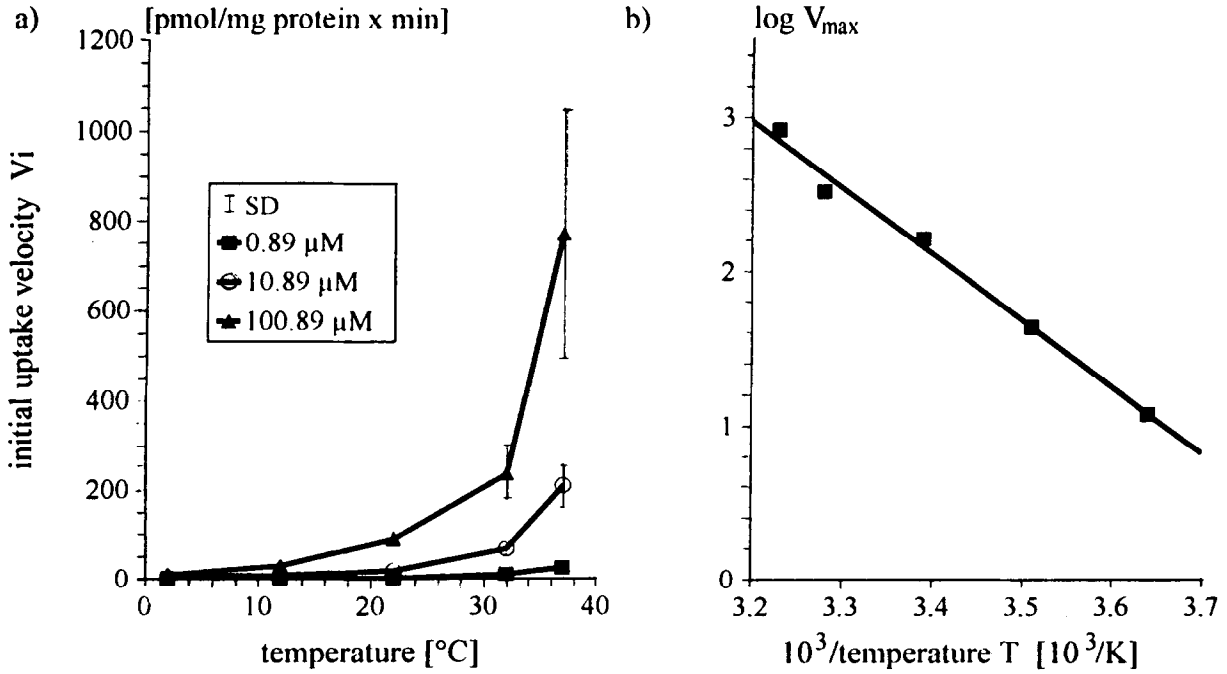


FIG. 5. Temperature dependency of CRC 220 uptake. (a) The uptake of 3 different CRC 220 concentrations in isolated rat liver cells was measured after 5-min preincubation at 2°C, 12°C, 22°C, 32°C, and 37°C. Initial uptake velocity was calculated from the linear part of the uptake. $n = 3$; mean ± SD. (b) Determination of the activation energy E of hepatocellular CRC 220 uptake in an Arrhenius diagram. By the Arrhenius equation, an activation energy E of 82 kJ/mol was calculated. $r = 0.995$.

centrations (0.89 μM and 10.89 μM) was significantly reduced (Fig. 7). At a concentration of 0.89 μM, the control initial uptake rate (V_i) was 31.1 ± 6.6 pmol/mg protein × min in sodium Tyrode buffer, whereas in choline and lithium Tyrode buffer, the V_i was 19.2 ± 3.8 and 18.7 ± 4.5 pmol/mg × min. The corresponding data at 10.89 μM CRC 220 were 203.6 ± 24.2 pmol/mg × min for control uptake, 131.1 ± 25.9 pmol/mg × min for uptake in choline buffer, and 157.2 ± 1.7 pmol/mg × min for uptake in lithium buffer (Table 1).

Effect of Chloride Ion Substitution on Hepatocellular CRC 220 Uptake

CRC 220 is a zwitterionic compound at pH 7.4 [14]. If a sodium ion is cotransported with a CRC 220 molecule, a positive charge enters the cells. Under these conditions, a more negative membrane potential should accelerate cell uptake. This was approached by replacement of chloride anions by nitrate and thiocyanate. The permeation of these lipophilic anions through the plasma membrane is more rapid because of their smaller solvation coat. Thus, they induce a more negative diffusion potential than chloride in the cell, thereby facilitating the entry of a positively charged substrate. In contrast, hydrophilic sulfate has a greater hydration coat and permeates more slowly than Cl^- through the membrane. Therefore, the inner side of the membrane is more positive and the entry of negatively charged substrates is facilitated [39]. Only the lipophilic anions nitrate and thiocyanate altered the CRC 220 uptake. Using two different thrombin inhibitor concentrations, the initial uptake velocity was increased by 39–66% (Fig. 7, Table 1).

CRC 220 uptake was not dependent upon extracellular calcium. In $CaCl_2$-free Tyrode buffer, uptake was the same as under control conditions (Fig. 7, Table 1).

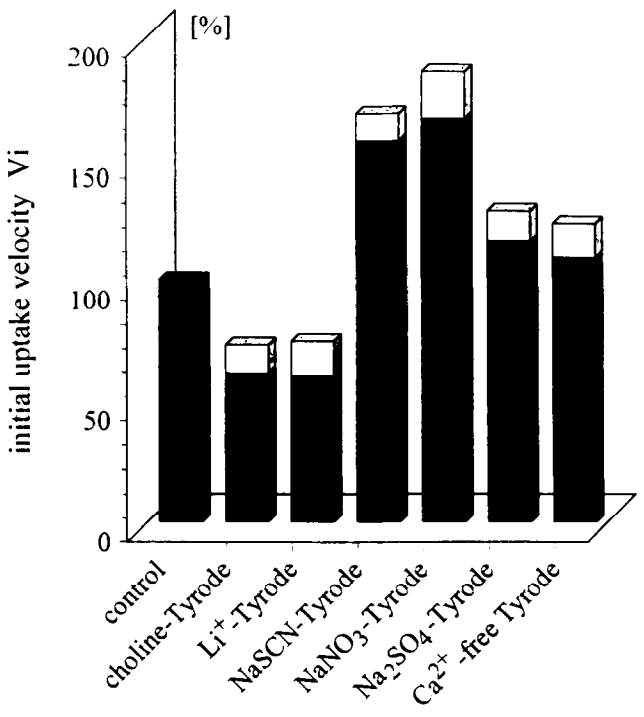


FIG. 7. Influence of sodium, chloride, and calcium ions on the uptake of CRC 220 at 37°C. Rat hepatocyte suspensions (2 mL) were preincubated in NaCl-Tyrode (control), choline-Tyrode, lithium-Tyrode, nitrate-Tyrode, thiocyanate-Tyrode, sulphate-Tyrode, and calcium-free Tyrode for 5 min before uptake of 0.89 μM [^{14}C]CRC 220 was measured. Initial velocity was determined from the linear part of the uptake. n = 3; mean ± SD.

Cell Specificity of CRC 220 Uptake and Binding by Nonvital Hepatocytes

The cell specificity of CRC 220 uptake was investigated in different cultured cell lines: the liver-derived FAO cells [33, 40, 41], HepG2 cells [31, 42, 43], HPCT 1E3 cells [34, 41], and HPCT 1E3TC cells [36] and the nonliver BHK (baby hamster kidney) cells [35]. Compared to isolated rat hepatocytes, the thrombin inhibitor did not accumulate in any of these cells (Fig. 8).

Nonvital hepatocytes bound CRC 220 rapidly. They were obtained by permeabilizing the cell membrane upon freezing and thawing. Binding was complete within 15 sec and no increase in cell-association radioactivity was observed during longer incubation, in contrast to effects in intact hepatocytes. The amount of cell-bound radioactivity was markedly low (20% of the maximal uptake value of intact hepatocytes) over a period of 10 min (Fig. 8).

Influence of Substrate Analogues

Competition studies were performed with isosteric derivatives containing aspartic acid, with the dipeptide framework Asp-Phe and Asp-Arg, as well as with the Asp-Phe methyl ester aspartame, to identify transport-relevant structural elements (Table 2). Furthermore, NAPAP, a potent thrombin inhibitor of the benzamidine type, which contains glycine instead of aspartic acid, was chosen because this compound lacks the acidic aspartic acid part [44]. The most potent inhibitor of CRC 220 uptake was CRC 369. The IC_{50} value was 1.9 ± 0.7 μM. This compound is an organic anion at physiological pH.

NAPAP decreased CRC 220 uptake only to a small degree that was not significant at a concentration of 20 μM. No inhibition took place with CRC 294, which is modified at the arylsulfonyl protecting group of the aspartic acid nitrogen. The dipeptides forming the structural framework of the thrombin inhibitor also had no inhibitory effects on CRC 220 uptake into rat hepatocytes (Table 2).

Competition Studies with Bile Acids, BSP, and Bumetanide

Different mono-, di-, and trihydroxylated bile acids, as well as BSP and bumetanide were examined for their inhibitory

TABLE 1. Influence of sodium, chloride, and calcium ions on the initial velocity of hepatocellular CRC 220 uptake

Incubation conditions	0.886 μM		10.886 μM	
	V_i [pmol × mg^{-1} × min^{-1}]	% of control	V_i [pmol × mg^{-1} × min^{-1}]	% of control
Control	31.1 ± 6.6	100	203.6 ± 24.2	100
Choline-Tyrode	19.2 ± 3.8	61.7 ± 17.9	131.1 ± 25.9	64.4 ± 14.9
Li^+-Tyrode	18.7 ± 4.5	60.1 ± 19.3	157.2 ± 1.7	77.2 ± 9.2
	P = 0.0119		P = 0.0091	
Control	25.5 ± 5.1	100	192.2 ± 7.1	100
NaSCN-Tyrode	40.1 ± 2.8	157.3 ± 33.3	316.6 ± 49.4	164.7 ± 26.4
$NaNO_3$-Tyrode	42.4 ± 5.0	166.3 ± 38.6	267.6 ± 57.1	139.2 ± 30.2
	P = 0.007		P = 0.0351	
Control	38.1 ± 4.1	100	211.3 ± 18.9	100
Na_2SO_4-Tyrode	44.2 ± 4.8	116.0 ± 17.7	192.8 ± 39.0	91.2 ± 20.2
	P = 0.1016		P = 0.4246	
Control	28.6 ± 2.3	100	203.9 ± 29.7	100
Ca^{2+}-free Tyrode	31.3 ± 4.1	109.4 ± 16.8	179.4 ± 35.0	88.0 ± 21.4
	P = 0.3775		P = 0.4077	

n = 3; $\bar{x}$ ± SD.

effect on [^{14}C]CRC 220 uptake into isolated rat hepatocytes (Fig. 9). The mono- and dihydroxylated bile acids were stronger inhibitors of [^{14}C]CRC 220 uptake than the trihydroxylated bile acids tested. BSP was also a potent inhibitor of hepatocellular CRC 220 uptake; the IC_{50} value amounted to 28.1 ± 3.9 μM. Bumetanide, however, decreased the transport of the thrombin inhibitor to only a small degree. Mutual transport inhibition was found between CRC 220 and cholate, as well as taurocholate. In the presence of sodium ions, CRC 220 uptake was inhibited by 100 μM of each bile acid to a comparable extent: at 29% by cholate and 31% by taurocholate. On the other hand, 100 μM CRC 220 inhibited cholate uptake in the presence of sodium ions to a significantly greater extent than taurocholate uptake (49% versus 20%) (Table 3). However, when sodium ions were absent, inhibition by 100 μM CRC 220 was more pronounced for taurocholate than for cholate uptake (64% vs 46%) (Table 3). The results indicated a common sodium-independent uptake of CRC 220, bile acids, and probably BSP. To support this attractive hypothesis, the CRC 220 analogue CRC 294, which lacked inhibition of CRC 220 transport, was tested vs bile acid uptake. Neither cholate nor taurocholate uptake was reduced by this compound.

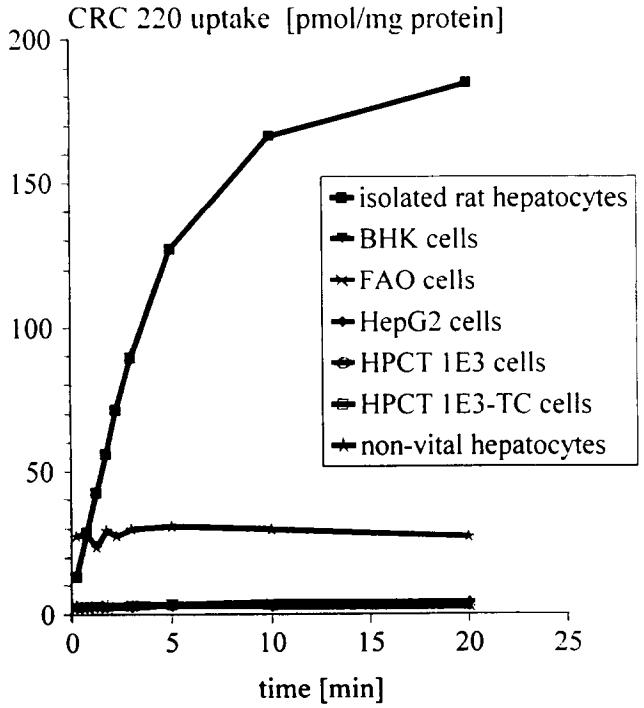


FIG. 8. Comparison of CRC 220 uptake in isolated rat hepatocytes, nonvital liver cells, and different cell culture lines. The uptake of 0.89 μM [^{14}C]CRC 220 was measured either in 1 mL of a rat hepatocyte suspension (2 × 10^6 cells/mL) or in suspensions of the cell culture lines. All cell suspensions correspond to a protein concentration of 4 mg/mL buffer. n = 3; mean ± SD.

DISCUSSION

The frequency of thromboembolic disorders, often leading to acute myocardial infarcts or stroke, has considerably increased in the last years. The serine protease, thrombin, plays an essential role in coagulation and platelet activation, so an effective control of the thrombin activity *in vivo* is urgently needed. Recently, a variety of highly potent thrombin inhibitors of low molecular weight were synthesized. The disadvantage of almost all of these peptide-based thrombin inhibitors, which considerably restricted their clinical use, is their short half-life time and low bioavailability, especially upon oral application [15–17].

CRC 220 is a specific thrombin inhibitor with sufficient absorption from the small intestine [13, 14]. However, CRC 220 is very rapidly eliminated into bile (Fig. 2). In the present study, we show that the hepatic clearance of the peptidomimetic thrombin inhibitor CRC 220 is due to an active carrier-mediated uptake process in the basolateral plasma membrane of rat hepatocytes.

TABLE 2. Influence of substrate analogues on the hepatocellular uptake of CRC 220

Substrate analogues	Concentration [μM]	V_i [pmol × mg^{-1} × min^{-1}]	% of control	P
–	control	23.6 ± 3.1	100	
CRC 220	20	10.7 ± 1.4	45.3 ± 8.4	≤0.0028
CRC 220	100	2.4 ± 1.4	10.2 ± 6.1	≤0.0001
	control	31.6 ± 5.6	100	
CRC 294	20	29.3 ± 4.5	92.7 ± 21.7	=0.5976
CRC 584	20	12.2 ± 3.1	38.6 ± 12.0	≤0.0001
CRC 569	20	10.5 ± 1.7	33.2 ± 8.0	≤0.0001
CRC 572	20	8.0 ± 1.9	25.3 ± 7.5	≤0.0001
CRC 369	20	6.2 ± 1.1	19.6 ± 4.9	≤0.0001
	control	21.5 ± 2.5	100	
Aspartame	20	21.0 ± 1.8	97.7 ± 14.1	≤0.8000
Aspartame	100	23.2 ± 2.9	107.9 ± 18.4	≤0.4828
NAPAP	20	16.2 ± 3.0	75.3 ± 16.5	≤0.0752
NAPAP	100	8.5 ± 0.9	39.5 ± 6.2	≤0.0001
	control	30.4 ± 1.3	100	
Asp-Phe	100	29.7 ± 2.3	97.7 ± 7.7	=0.6633
Asp-Arg	100	28.0 ± 1.5	92.1 ± 6.3	=0.0982

Aliquots of 2 mL hepatocyte suspension were incubated with 20 μM or 100 μM of the inhibitors prior to the addition of 0.89 μM [^{14}C]CRC 220. Initial uptake velocity was calculated between 15 and 105 sec. n, 3; mean ± SD.

Isolated rat hepatocytes accumulate the amphiphilic thrombin inhibitor 25–125 fold at 37°C. Only a very small part of this cellular uptake is due to passive diffusion. The permeability coefficient of this part is, so far, the lowest found for compounds transported into hepatocytes [45].

Because diffusion is so low, cells in addition to hepatocytes lack significant uptake of CRC 220. Hepatoma cells (HepG2, FAO hepatoma cells) and BHK cells, which are transport negative regarding bile acids [34, 41, 42, 46] further lack CRC 220 uptake (Fig. 8). It appears that the distribution of CRC 220 in the organism is determined by carrier-mediated clearance processes in the liver (Fig. 4). Bile acids markedly reduce CRC 220 liver clearance when applied simultaneously to anesthetized rats (Fig. 2). Bile acids also diminish uptake of [^{14}C]CRC 220 into isolated hepatocytes (Fig. 9). We, therefore, conclude that CRC 220 is transported from blood into the cell by a transport system for bile acids.

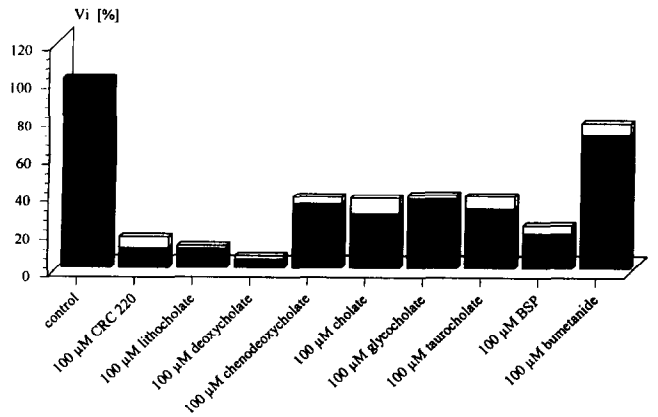


FIG. 9. Inhibition of CRC 220 uptake into isolated rat hepatocytes by different mono-, di-, and trihydroxylated bile acids, BSP, and bumetanide. Aliquots of 2 mL liver cell suspension were incubated with the indicated concentrations of inhibitors for 30 sec prior to the addition of 0.89 μM [^{14}C]CRC 220. The initial uptake velocity was determined between 15 and 105 sec. *n* = 3; mean ± SD.

Recently, two bile acid carriers from the basolateral plasma membrane of rat liver cells were cloned by means of functional expression cloning in *Xenopus laevis* oocytes [47, 48]. The first system is a Na^+/taurocholate-cotransporting polypeptide Ntcp, which predominantly transports taurocholate. This transport is highly Na^+-dependent [47, 49]. The second system known as the organic anion transporting polypeptide oatp allows Na^+-independent uptake of various bile acids as well as other organic anions [48, 49]. A substrate of the oatp is BSP.

The Ntcp was recently transfected into a liver-derived cell line, HPCT, which stably expressed sodium-dependent taurocholate uptake [36]. With the clone HPCT 1E3-TC, we directly examined a possible transport of CRC 220 *via* Ntcp. However, no transport occurred (Fig. 8).

On the other hand, BSP is among the most potent inhibitors of CRC 220 uptake (IC_{50} = 28.1 μM), whereas inhibition by bumetanide is not significant (Fig. 9). These results indicate that uptake of CRC 220 *via* oatp is likely. This would mean that this transporter does not only perform uptake of bile acids and cholephilic organic anions, but also of a modified dipeptide. Oatp, however, would only allow sodium-independent CRC 220 uptake; because, in the isolated liver cell model, CRC 220 uptake was partly sodium-dependent, an additional uptake mechanism is

TABLE 3. Inhibition of hepatocellular [^{3}H]cholic acid/cholate and [^{3}H]taurocholic acid/taurocholate uptake by CRC 220 in sodium- and choline-Tyrode buffer

Uptake buffer	Transport substrate	CRC 220 conc. (μM)	V_i (pmol × mg^{-1} × min^{-1})	% of control
Na^+-Tyrode	cholate	control	280.1 ± 48.7	100
		100	142.2 ± 30.0	50.8 ± 13.9
			$P = 0.0029$	
Na^+-Tyrode	taurocholate	control	789.6 ± 43.0	100
		100	630.9 ± 103.3	79.9 ± 13.8
			$P = 0.07$	
Choline-Tyrode	cholate	control	121.7 ± 15.4	100
		100	66.3 ± 10.0	54.4 ± 10.7
			$P = 0.0043$	
Choline-Tyrode	taurocholate	control	79.9 ± 7.8	100
		100	27.6 ± 1.2	35.8 ± 3.7
			$P \leq 0.0001$	

Aliquots of 2 mL hepatocyte suspension were preincubated with 100 μM CRC 220 for 30 sec before uptake of 40 nM/10 μM [^{3}H]cholic acid/cholate or 40 nM/10 μM [^{3}H]taurocholic acid/taurocholate was measured. Initial velocity was determined between 15 and 105 sec. n = 4; mean ± SD.

likely. This carrier for sodium-dependent CRC 220 transport remains unknown.

The most potent inhibitor of CRC 220 transport is CRC 369, which lacks the positively charged amidino group. At pH 7.4, this substance is an anionic compound. This is in line with our conclusion that a transport system for organic anions is responsible for CRC 220 uptake. Correspondingly, substances lacking the negatively charged carboxylic group should not, or only slightly, decrease CRC 220 uptake. That applies to the most potent thrombin inhibitor of the benzamidine type, NAPAP, which contains glycine instead of aspartic acid [44, 50, 51].

In contrast to CRC 369, CRC 294, which is modified at the arylsulfonyl protecting group of the aspartic acid nitrogen but is also a zwitterion, does not inhibit CRC 220 uptake. In addition to the lack of uptake competition by the dipeptides forming the structural framework, this underlines that the hepatic CRC 220 carrier is not a transport system for dipeptides. Therefore, the experiments indicate that—apart from the acidic moiety—the substituted functional groups, especially the arylsulfonyl protecting group of the aspartic acid nitrogen, form a favorable structure for hepatic uptake (Table 2).

In summary, the thrombin inhibitor CRC 220 is taken up into rat liver cells *via* an active carrier-mediated transport process. The transport is liver-specific and closely related to the transport of taurocholate, cholate, and BSP. The cloned Na^+/taurocholate cotransporting polypeptide Ntcp is not involved in the uptake of CRC 220. It is very likely that oatp participates in the transport of this peptide-based thrombin inhibitor. To improve the bioavailability of this class of thrombin inhibitors, their carrier-mediated hepatocellular clearance must be reduced. Molecules with lower affinity to the transporter, but conserved affinity to thrombin, need to be developed.

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References

1. Wünsch E, Peptide factors as pharmaceuticals: criteria for application. *Biopolymers* **22:** 493–505, 1983.
2. Illingworth DR, Clinical implications of new drugs for lowering plasma cholesterol concentrations. *Drugs* **41:** 151–160, 1991.
3. Kathawala FG, HMG-CoA reductase inhibitors: an exciting development in the treatment of hyperlipoproteinemia. *Med Res Rev* **11:** 121–146, 1991.
4. Aronson JK and Ng LL, HMG CoA reductase inhibition in the treatment of hypercholesterolaemias. *Q J Med* **74:** 111–115, 1990.
5. Hruby VJ, Design of peptide hormone and neurotransmitter analogues. *Trends Pharmacol Sci* **6:** 259–263, 1985.
6. Hui KY, Holtzman EJ, Quinones MA, Hollenbeg NK and Haber E, Design of renin inhibitory peptides. *J Med Chem* **31:** 1679–1686, 1988.
7. Kramer W, Dechent C, Girbig F, Gutjahr U and Neubauer H, Intestinal uptake of dipeptides and β-lactam antibiotics. I. The intestinal uptake system for dipeptides and β-lactam antibiotics is not part of a brush border membrane peptidase. *Biochem Biophys Acta* **1030:** 41–49, 1990.
8. Hilpert K, Ackermann J, Banner DW, Gast A, Gubernator K, Hadvary P, Labler L, Muller K, Schmid G, Tschopp TB, *et al.* Design and synthesis of potent and highly selective thrombin inhibitors. *J Med Chem* **37:** 3889–3901, 1994.
9. Romisch J, Diehl KH, Hoffmann D, Krahl-Mateblowski U, Reers M, Stüber W and Paques EP, Comparison of in vitro and in vivo properties of r-hirudin (HBW 023) and a synthetic analogous peptide. *Haemostasis* **23:** 249–258, 1993.
10. Meijer DKF, Transport and metabolism in the hepatobiliary system. In: *Handbook of Physiology Vol. III, The Gastrointestinal System* (Eds. Schultz SG, Forte JG and Rauner BB), Chap. 35, pp. 717–758. American Physiological Society, Bethesda, MD, 1989.
11. Ziegler K, Hepatocellular transport of cyclic and linear peptides. In: *Hepatic endo- and xenobiotic metabolism* (Eds. Bock KW, Gerok W, Matern S and Schmid R), Chap. 31, pp.

287–302. Kluwer Academic Publishers, Dordrecht, Boston, London, 1991.
12. Stüber W, Koschinsky R, Kolar C, Reers M, Dickneite G, Hoffmann D, Czech J, Diehl K-H and Pâques E-P, Inhibition of thrombin by derivatives of the dipeptide asparticacid-amidinophenylalanine. In: *Peptides, chemistry, structure and biology: Proceedings of the 13th American Peptide Symposium* (Eds. Hodges RS and Smith JA), pp. 643–645. ESCOM Leiden, Edmonton, AL, Canada, 1994.
13. Reers M, Koschinsky R, Dickneite G, Hoffmann D, Czech J and Stüber W, Synthesis and characterisation of novel thrombin inhibitors based on 4-amidinophenylalanine. *J Enzyme Inhibition* **9:** 61–72, 1995.
14. Walter E, Kissel T, Reers M, Dickneite G, Hoffmann D and Stüber W, Transepithelial transport properties of peptidomimetic thrombin inhibitors in monolayer of a human intestinal cell line (Caco-2) and their correlation to in vivo data. *Pharm Res* **12:** 360–365, 1995.
15. Tapparelli C, Metternich R, Ehrhardt C and Cook NS, Synthetic low-molecular weight thrombin inhibitors: molecular design and pharmacological profile. *Trends Pharmacol Sci* **14:** 366–376, 1993.
16. Hauptmann J, Kaiser B, Paintz M and Markwardt F, Biliary excretion of synthetic benzamidine-type thrombin inhibitors in rabbits and rats. *Biomed Biochim Acta* **46:** 445–453, 1987.
17. Kaiser B, Hauptmann J and Markwardt F, Studies on toxicity and pharmacokinetics of the synthetic thrombin inhibitor D-phenylalanyl-L-prolyl-L-arginine nitrile. *Pharmazie* **46:** 131–134, 1991.
18. Bagdy D, Szabó G, Barabas E and Bajusz S, Inhibition by D-Me Phe-Pro-Arg-H (GYKI 14,766) of thrombus growth in experimental models of thrombosis. *Thromb Haemostasis* **68:** 125–129, 1992.
19. Balasubramanian N, St Laurent NA, Wright JJ, Schumacher WA and Seiler SM, Active site-directed synthetic thrombin inhibitors: synthesis, in vitro and in vivo activity profile of BMY 44621 and analogs. An examination of the role of the amino group in the D-Phe-Pro-Arg-H series. *J Med Chem* **36:**300–303, 1993.
20. Ziegler K, Lins W and Frimmer M, Hepatocellular transport of cyclosomatostatins: evidence for a carrier system related to the multispecific bile acid transporter. *Biochim Biophys Acta* **1061:** 287–296, 1991.
21. Bertrams A and Ziegler K, Hepatocellular uptake of peptides by bile acid transporters: relationship of carrier-mediated transport of linear peptides with renin-inhibiting activity to multispecific bile acid carriers. *Biochim Biophys Acta* **1091:** 337–348, 1991.
22. Petzinger E and Frimmer M, Energy linked uptake of demethylphalloin by isolated rat liver cells. *Naunyn-Schmiedeberg's Arch Pharmacol* **319:** 87–92, 1982.
23. Petzinger E, Ziegler K and Frimmer M, Occurrence of a multispecific transporter for the hepatocellular accumulation of bile acids and various cyclopeptides. In: *Bile Acids and the Liver* (Eds. Paumgartner G, Stiehl A and Gerok W), pp. 111–124. MTP-Press, Lancaster, U.K. 1987.
24. Berry MN and Friend DS, High-yield preparation of isolated rat liver parenchymal cells. *J Cell Biol* **43:** 506–520, 1969.
25. Petzinger E and Seeger R, Scanning electron microscopic studies on the cytolytic effect of phallolysin on isolated rat hepatocytes and AS-30D hepatoma cells. *Naunyn-Schmiedeberg's Arch Pharmacol* **295:** 211–213, 1976.
26. Bradford MM, A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:** 248–254, 1976.
27. Klingenberg M and Pfaff E, Means of terminating reaction. *Methods Enzymol* **10:** 680–684, 1967.
28. Müller N and Petzinger E, Hepatocellular uptake of aflatoxin B_1 by non-ionic diffusion. Inhibition of bile acid transport by interference with membrane lipids. *Biochim Biophys Acta* **938:** 334–344, 1988.
29. Drochmans P, Wanson JC and Mosselmans R, Isolation and subfractionation on ficoll gradients of adult rat hepatocytes. *J Cell Biol* **66:** 1–22, 1975.
30. LeCam A and Freychet P, Neutral amino acid transport. *J Biol Chem* **252:** 148–156, 1977.
31. Aden DP, Fogel A, Plotkin S, Damjanov J and Knowles BB, Controlled synthesis of HBsAg in a differentiated human liver carcinoma-derived cell line. *Nature* **282:** 615–616, 1979.
32. Reuber MD, A transplantable bile-secreting hepatocellular carcinoma in the rat. *J Natl Cancer Inst* **26:** 891–899, 1961.
33. Deschatrette J and Weiss MC, Characterization of differentiated and dedifferentiated clones from a rat hepatoma. *Biochimie* **56:** 1603–1611, 1974.
34. Petzinger E, Föllmann W, Blumrich M, Walther P, Hentschel J, Bette P, Maurice M and Feldmann G, Immortalization of rat hepatocytes by fusion with hepatoma cells. I. Cloning of a hepatocytoma cell line with bile canaliculi. *Eur J Cell Biol* **64:** 328–338, 1994.
35. Stoker M and MacPherson I, Studies on transformation of hamster cells by polyoma virus in vitro. *Virology* **14:** 359–370, 1961.
36. Platte HD, Honscha W, Schuh K and Petzinger E, Stable transfection of the hepatic sodium dependent taurocholate transporter into an immortalized liver-derived cell line and V79 fibroblasts. *Eur J Cell Biol*, **70:** 54–60, 1996.
37. Höfer M, *Transport durch biologische Membranen*. Verlag Chemie, Weinheim, New York, 1977.
38. Petzinger E and Fischer K, Transport functions of the liver. Lack of correlation between hepatocellular ouabain uptake and binding to (Na^+-K^+)-ATPase. *Biochim Biophys Acta* **815:**334–340, 1985.
39. Saktor B, Electrogenic and electroneutral Na^+ gradient dependent transport system in the renal brush border membrane vesicle. *Curr Top Membranes Transp* **13:** 291–300, 1980.
40. Von Dippe P and Levy D, Characterization of the bile acid transport system in normal and transformed hepatocytes. *J Biol Chem* **258:** 8896–8901, 1983.
41. Blumrich M, Zeyen-Blumrich U, Pagels P and Petzinger E, Immortalization of rat hepatocytes by fusion with hepatoma cells. II. Studies on the transport and synthesis of bile acids in hepatocytoma (HPCT) cells. *Eur J Cell Biol* **64:** 339–347, 1994.
42. Min AD, Goeser T, Liu R, Champbell CE, Novikoff PM and Wolkoff AW, Organic anion transport in HepG2 cells: absence of the high-affinity, chloride-dependent transporter. *Hepatology* **14:** 1217–1223, 1991.
43. Ziegler K, Blumrich M and Hummelsiep S, The transport for the HMG-CoA reductase inhibitor pravastatin is not present in HepG2 cells. Evidence for the nonidentity of the carrier for pravastatin and certain transport systems for BSP. *Biochim Biophys Acta* **1223:** 195–201, 1994.
44. Stürzebecher J, Markwardt F, Voigt B, Wagner G and Walsmann P, Cyclic amides of Nα-arylsulfonylaminoacylated 4-amidinophenylalanine—tight binding inhibitors of thrombin. *Thromb Res* **29:** 635–642, 1983.
45. Petzinger E, Transport of organic anions in the liver. An update on bile acid, fatty acid, monocarboxylate, anionic amino acid, cholephilic organic anion and anionic drug transport. *Rev Physiol Biochem Pharmacol* **123:** 47–211, 1994.
46. Marchegiano P, Carubbi F, Tiribelli C, Amarri S, Stebel M, Lunazzi GC, Levy D and Bellentani S, Transport of sulfobromophthalein and taurocholate in the HepG2 cell line in relation to the expression of membrane carrier proteins. *Biochem Biophys Res Commun* **183:** 1203–1208, 1992.

47. Hagenbuch B, Stieger B, Foguet M, Lübbert H and Meier PJ, Functional expression cloning and characterization of the hepatocyte Na^+/bile acid cotransport system. *Proc Natl Acad Sci* **88:** 10629–10633, 1991.
48. Jaquemin E, Hagenbuch B, Stieger B, Wolkoff AW and Meier PJ, Expression cloning of a sodium-independent organic anion uptake system of rat liver. *Proc Natl Acad Sci* **91:** 133–137, 1994.
49. Hagenbuch B, Jaquemin E and Meier PJ, Na^+-dependent and Na^+-independent bile acid uptake systems in the liver. *Cell Physiol Biochem* **4:** 198–205, 1994.
50. Stürzebecher J, Prasa D, Bretschneider E, Bode W, Bauer M, Brandstetter H, Wikström P and Vieweg H, New developments in the field of benzamidine-derived thrombin inhibitors. In: *DIC: Pathogenesis, Diagnosis and Therapy of Disseminated Intravascular Fibrin Formation* (Eds. Müller-Berghaus G, Madlener K, Blombäck M and ten Cate JM), pp. 183–190. Excerpta Medica, Amsterdam, London, New York, Tokyo, 1993.
51. Stürzebecher J, Prasa D, Wikström P and Vieweg H, Structure-activity relationships of inhibitors derived from 3-amidinophenylalanine. *J Enzyme Inhib* **9:** 87–99, 1995.