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Differential inhibition by cyclosporins of primary-active ATP-dependent transporters in the hepatocyte canalicular membrane

Matthias Böhme, Markus Büchler, Michael Müller, Dietrich Keppler*

Division of Tumor Biochemistry, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 280, D-69120 Heidelberg, Germany

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The distinct ATP-dependent transporters for taurocholate, leukotriene C4, and daunorubicin, studied in rat liver canalicular membrane vesicles, are sensitive to inhibition by cyclosporin A and its non-immunosuppressive analog PSC 833. K_i values for cyclosporin A were 0.2, 3.4 and 1.5 μM for the transport of taurocholate, leukotriene C4, and daunorubicin, respectively. The corresponding K_i values for PSC 833 were 0.6, 29, and 0.3 μM. Both inhibitors were competitive with respect to the three substrates. The cyclosporins serve as new and potent tools to interfere with different potency with the distinct ATP-dependent export carriers in the hepatocyte canalicular membrane.

ATP-dependent transport; Daunorubicin; LTC4; Multidrug resistance; *P*-glycoprotein; Taurocholate

1. INTRODUCTION

Primary-active ATP-dependent transport is the major mechanism to secrete toxic and non-toxic, exogenous as well as endogenous substances across the hepatocyte canalicular membrane into bile [1]. Three distinct ATP-dependent carriers were so far described in this membrane domain. (i) The ATP-driven bile salt export carrier (BSEC), discovered in 1991, is now considered to be predominantly responsible for the secretion of bile salts across the canalicular membrane [2–5]. (ii) The ATP-dependent leukotriene export carrier (LTEC) secretes conjugates of various exogenous and endogenous substances, such as the cysteinyl leukotrienes, with glutathione, glucuronide, or sulfate moieties [1,6]. (iii) The ATP-dependent daunorubicin transport mediated by *P*-glycoprotein was established in analogy to its function in multidrug-resistant tumor cells, although endogenous substrates for this export carrier still await identification [7,8]. The ATP-dependent daunorubicin transport in liver showed characteristic properties of a *P*-glycoprotein-mediated transport including inhibition by verapamil [8]. In contrast, BSEC and LTEC are not inhibited by typical *P*-glycoprotein substrates [2,6]. The only common inhibitor of these P-type ATPases described previously has been vanadate [2–8].

Cyclosporin A (CsA) and its non-immunosuppressive analog PSC 833 are potent drugs to reverse the multidrug resistance (MDR) phenotype in tumor cells expressing high levels of the *mdr1* gene product *P*-glycoprotein [9]. In vitro studies using isolated plasma membrane vesicles from multidrug resistant carcinoma cell lines have shown that CsA is a strong inhibitor of *P*-glycoprotein-mediated, ATP-driven vinblastine transport [10]. In cytotoxicity assays and in vivo studies in mice, CsA and its analog PSC 833 were capable to overcome the resistance of *P*-glycoprotein-expressing cells and implanted tumors to a variety of chemotherapeutic agents [11,12]. In human serum levels of 2–4 μM CsA prolonged the elimination half-life of the co-administered chemotherapeutic drug more than two times [13]. A possible explanation is that cyclosporins do not only mediate an enhanced accumulation of the co-administered drug in tumor cells, but also delay the drug elimination into bile, possibly by inhibiting *P*-glycoprotein-mediated transport in the liver [13]. We present evidence that this observation is based on the potent inhibition of ATP-dependent daunorubicin transport in the hepatocyte canalicular membrane.

This study defines the type and extent of inhibition by cyclosporins of the different ATP-dependent transporters using highly enriched rat hepatocyte canalicular membrane vesicles. These data provide information on possible side effects during cyclosporin therapy either for immunosuppression or drug resistance modification. In addition, cyclosporins appear as new and potent tools for inhibition of different ATP-dependent export carriers.

2. MATERIALS AND METHODS

2.1. *Chemicals and inhibitors*

ATP (potassium salt), 5′-AMP (free acid), creatine phosphate (Tris salt), unlabeled taurocholate, and daunorubicin were purchased from

*Corresponding author. Fax: (49) (6221) 42-2402.

Abbreviations: BSEC, bile salt export carrier; CsA, cyclosporin A; IC_{50}, concentration required for 50% inhibition; LTEC, leukotriene export carrier; MDEC, multidrug export carrier; MDR, multidrug resistance; SDZ PSC 833 or PSC 833, (3′-oxo-4-butenyl-4-methyl-threonine1)-(Val^2)-cyclosporin; TC, taurocholate; LTC4, leukotriene C4.

Sigma Chemical Co., St. Louis, MO, USA. Creatine kinase and reduced glutathione were obtained from Boehringer-Mannheim, Mannheim, Germany. Unlabeled LTC4 was from Amersham-Buchler, Braunschweig, Germany. [γ-^{3}H]Daunorubicin (59 GBq/mmol), [γ-^{3}H]taurocholate (74 GBq/mmol), and [14,15,19,20-^{3}H]LTC4 (4700 GBq/mmol) were from DuPont-New England Nuclear, Boston, MA, USA. Nitrocellulose filters (pore size 0.2 μm) were from Schleicher & Schüll, Dassel, Germany. Nick spin columns filled with Sephadex G-50 fine were purchased from Pharmacia-LKB, Freiburg, Germany. Scintillation fluids (Filter Count and Ultima Gold) were from Canberra Packard, Warrenville, IL, USA. All other chemicals were of analytical grade. CsA and PSC 833, kindly provided by Sandoz AG, Basel, Switzerland, were prepared as 10 mM stock solutions in 100% ethanol and stored at −20°C. Both were diluted to their final concentration in 250 mM sucrose and 10 mM HEPES/Tris (pH 7.4), so that the final ethanol concentration never exceeded 0.5%. In control experiments without CsA or PSC 833 a corresponding concentration of ethanol was added to the medium in order to control any effects of the solvent.

2.2. *Animals*

For preparations enriched in liver canalicular membranes, male Wistar rats (200–250 g) were obtained from the Zentralinstitut für Versuchstierzucht, Hannover, Germany. Animals were maintained on a standard diet with free access to food and water.

2.3. *Preparation and characterization of membranes*

Liver membrane fractions enriched in the hepatocyte canalicular domain were prepared and characterized as described [2].

2.4. *Measurement of ATP-dependent taurocholate transport into inside-out canalicular membrane vesicles*

Transport of [^{3}H]TC into the vesicles [2,6] was measured as follows: membrane vesicles (30 μg of protein) were incubated in the presence of 4 mM ATP, 10 mM $MgCl_2$, 10 mM creatine phosphate, 100 μg/ml creatine kinase, and the labeled substrate in 250 mM sucrose and 10 mM HEPES/Tris (pH 7.4) at a final volume of 110 μl. Potassium or Tris salts instead of sodium salts of the medium components were used throughout. 20-μl samples were taken at the indicated time points and diluted in 1 ml of ice-cold 250 mM sucrose and 10 mM HEPES/Tris (pH 7.4). The samples were applied to nitrocellulose filters (0.2 μm pore size) pre-soaked in 250 mM sucrose and 10 mM HEPES/Tris (pH 7.4). Uptake of [^{3}H]TC was measured by a rapid filtration technique [2–6]. The nitrocellulose filters were rinsed with 5 ml of 1 mM unlabeled TC, 250 mM sucrose, 10 mM HEPES/Tris (pH 7.4) and 5 ml of 250 mM sucrose, 10 mM HEPES/Tris (pH 7.4). In control experiments ATP was replaced by 5'-AMP. Vesicle-associated radioactivity retained on the filters was assessed by liquid scintillation counting. Transport rates were calculated by subtracting the values in the presence of 5'-AMP from the ones in the presence of ATP.

2.5. *Measurement of ATP-dependent LTC4 transport into canalicular membrane vesicles*

[^{3}H]LTC4 transport was essentially determined as described above for [^{3}H]TC transport. Reduced glutathione was added to the incubation at a final concentration of 5 mM to prevent binding of LTC4 to the membrane-bound glutathione *S*-transferase and degradation of LTC4 to LTD4 by the canalicular membrane γ-glutamyltransferase.

2.6. *Measurement of ATP-dependent daunorubicin transport into canalicular membrane vesicles*

The incubation to measure daunorubicin transport into canalicular membrane vesicles was similar as described above for the other substrates. The rapid filtration method with nitrocellulose filters was replaced, however, by centrifugation of the vesicles through a gel matrix. Nick spin columns (1 g Sephadex G-50/2 ml) were prepared by rinsing with 1 mM unlabeled TC, 250 mM sucrose, 10 mM HEPES/Tris (pH 7.4), and centrifuged at 400 × *g* and 4°C for 4 min before use. The incubation medium was composed of the same ATP-regenerating system as for TC transport in a final volume of 110 μl. 20-μl samples of the incubation medium were diluted in 80 μl of ice-cold 250 mM sucrose, 10 mM HEPES/Tris (pH 7.4) and loaded on the Sephadex G-50 column. The columns were rinsed with 100 μl of 250 mM sucrose, 10 mM HEPES/Tris (pH 7.4) and centrifuged at 400 × *g* and 4°C for 4 min as described [14,15]. The effluent was collected and assayed for the radioactivity associated with the vesicles.

3. RESULTS

3.1. *Kinetic characterization of the inhibition by cyclosporins of the primary-active transport of taurocholate, leukotriene C_4, and daunorubicin*

CsA and PSC 833 both inhibited the ATP-dependent TC transport efficiently with IC50 values of 1 μM (Figs. 1 and 2). The concentrations of CsA and PSC 833 necessary to induce 50% inhibition of ATP-dependent LTC4 transport were 6-times and 15-times higher, respectively, than the ones required to inhibit TC transport by 50% (Figs. 1 and 2). ATP-driven daunorubicin transport exhibited a marked discrepancy between the inhibitory potency of CsA and PSC 833, i.e. a 5-times less potent inhibition by CsA as compared to PSC 833 (Figs. 1 and 2).

Kinetic analyses indicated a competitive inhibition by both CsA and PSC 833 of LTC4 as well as daunorubicin transport (Figs. 3 and 4). TC transport was also competitively inhibited by PSC 833 (graph not shown), which corresponds to our report on inhibition of ATP-dependent TC transport by CsA [16]. The K_i values for TC transport were similar for CsA and PSC 833 (Table I). The corresponding K_i values for inhibition of LTC4 transport showed the largest difference of inhibition between the two cyclosporins (Fig. 3). The K_i values for daunorubicin transport indicated a 5-fold difference of transport inhibition by the two cyclosporins (Fig. 4).

3.2. *Comparison of substrate and inhibitor efficiency of the three transporters in the canalicular membrane*

The LTEC showed the lowest K_m value of the three

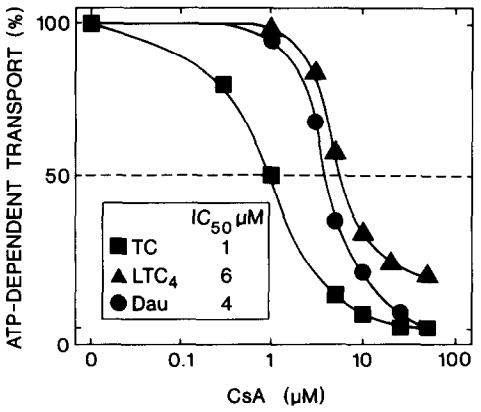

Fig. 1. Inhibition by CsA of ATP-dependent transport of TC, LTC4, and daunorubicin into isolated rat canalicular membrane vesicles. Membrane vesicles were incubated in the presence of ATP, $MgCl_2$, creatine phosphate, creatine kinase, and the respective substrate for 1 min. In the blank assays ATP was replaced by 5'-AMP. The concentrations of TC, LTC4, and daunorubicin were 5 μM, 50 nM, and 5 μM, respectively. Each point represents the mean from 4 separate measurements.

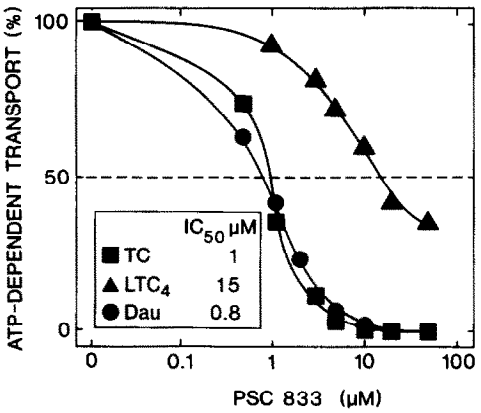

Fig. 2. Inhibition by PSC 833 of ATP-dependent transport of TC, LTC4, and daunorubicin into isolated canalicular membrane vesicles. Incubation conditions were the same as described in the legend to Fig. 1. Each point represents the mean from 4 separate measurements.

carriers with 0.32 μM for LTC4 followed by the BSEC with a K_m value of 10 μM for TC. The MDEC displayed the highest K_m value of 20 μM as measured with daunorubicin, in the absence of a known endogenous substrate (Table II). Calculation of the V_{max}/K_m ratios for the transporters, which provides information on the efficiency of a transporter with a given substrate, leaves the BSEC and LTEC as the most efficient with ratios of 180 and 425, respectively, compared to MDEC with a ratio of only 14 (Table II).

The efficiency of the cyclosporins as inhibitors in the presence of a given substrate was calculated by the K_m/K_i ratio (Table I). The most conspicuous result was the limited effect of the two cyclosporins on the LTEC (ratio 0.09 and 0.01 for CsA and PSC 833, respectively), while the ratio for CsA is similar for the BSEC and MDEC (25 and 13, respectively). PSC 833 achieves the highest inhibitor efficiency on the MDEC (ratio 67) compared to the BSEC (ratio 17; Table II).

4. DISCUSSION

4.1. *Differential inhibition of the primary-active transporters for taurocholate, LTC_4, and daunorubicin*

The distinct ATP-dependent transporters for the physiological substrates TC [2–5] and LTC4 [6], and for the chemotherapeutic drug daunorubicin [8] in the hepatocyte canalicular membrane exhibit different susceptibility to inhibition by two cyclosporins which differ in their immunosuppressive action. PSC 833 was a more potent inhibitor of multidrug transport than CsA. ATP-dependent TC transport was equally affected by CsA and PSC 833. Though the Ki values for inhibition of TC and daunorubicin transport by PSC 833 are similar, the inhibitor efficiency, estimated from the K_m/K_i ratios, revealed the 4-fold stronger influence on daunorubicin transport (Table I). Daunorubicin transport showed a low substrate efficiency ratio, which may be due to the fact that it is an unphysiologic exogenous substrate for this transporter.

Table I

K_i values for CsA and PSC 833 and inhibitor efficiency relative to the substrate

Substrate for ATP-dependent transport	K_i		K_m/K_i	
	CsA	PSC	CsA	PSC
	(μM)			
Taurocholate	0.2	0.6	50	17
LTC_4	3.4	29	0.09	0.01
Daunorubicin	1.5	0.3	13	67

The concentration of TC ranged from 1 μM to 20 μM, from 25 nM to 500 nM for LTC4, and from 1 μM to 10 μM for daunorubicin. Transport studies were performed as described in section 2. K_i values were determined by the Lineweaver-Burk equation for competitive inhibition. Values are from quadruplicate determinations with all standard deviations at less than 15% of the mean.

The non-immunosuppressive CsA derivative PSC 833 is modified in only 3 positions [11,12]. At the first amino acid CsA (*N*-methyl-4-butenyl-4-methyl-threonine) the *N*-methyl group is removed and the hydroxyl group at carbon atom 3 is oxidized to an oxo group. The second amino acid (L-2-amino-butyrate) is replaced by valine, resulting in an additional methyl group. These modifications lead to a loss of immunosuppressive properties [12]. In our study, we demonstrate that PSC 833 gains a higher efficiency to inhibit the *P*-glycoprotein-mediated daunorubicin transport while losing efficiency to

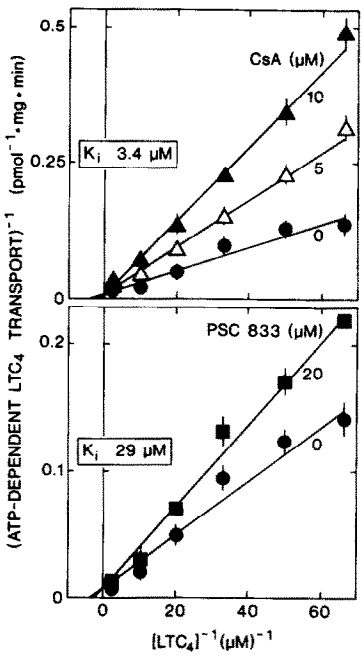

Fig. 3. Competitive inhibition by CsA (upper panel) and PSC 833 (lower panel) of ATP-dependent LTC4 transport. Double reciprocal plots according to Lineweaver and Burk in the presence of 5 μM and 10 μM CsA (upper panel) and 20 μM PSC 833 (lower panel) with varying LTC4 concentrations (25–50 nM). Incubation conditions were the same as described in the legend to Fig. 1. The straight lines were calculated by the least squares method. Mean values ± S.D. from 4 determinations.

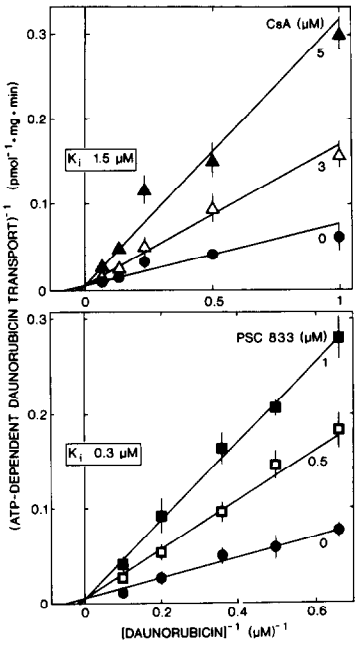

Fig. 4. Competitive inhibition by CsA (upper panel) and PSC 833 (lower panel) of ATP-dependent daunorubicin transport. Double reciprocal plots in the presence of 3 μM and 5 μM CsA (upper panel) and 0.5 μM and 1 μM PSC 833 (lower panel) with daunorubicin concentrations ranging from 1 to 10 μM. The lines are calculated by the least squares method. Mean values ± S.D. from 4 determinations.

inhibit LTC4 transport (Figs. 1 and 2). The results demonstrate how cyclosporin derivatives may be used as molecular tools to characterize transport processes in an in vitro membrane vesicle transport system. Especially CsA and PSC 833 in combination provide a means to distinguish between transporters by their inhibitor efficiency.

4.2. *Pathophysiological implications*

Inhibition of the ATP-dependent transport of organic compounds may have several pathophysiological implications: (i) cholestasis indicated by a rise in serum bile salts and bilirubin is a well known side effect of immunosuppressive CsA therapy after successful organ transplantation [17,18]. Since CsA and PSC 833 strongly inhibit the BSEC, it is likely that inhibition of the ATP-dependent bile salt transporter in liver is the underlying molecular mechanism. (ii) Inhibition of primary-active ATP-dependent daunorubicin transport may not only serve in the modification of multidrug resistance, but also as a mechanism to delay the elimination of active drugs from the organism by the liver [13]. (iii) Members of the ATP-binding cassette superfamily [19,20] other than *P*-glycoprotein may also be targets for inhibition by cyclosporins [10,16]. This mechanism can be useful to define the function of these transporters by in vivo measurements of substance secretion as well as in vitro membrane vesicle transport assays.

Table II

Kinetic parameters of three distinct transporters in the hepatocyte canalicular membrane

Substrate for ATP-dependent transport	V_{max} (pmol · mg^{-1} · min^{-1})	K_m (μM)	V_{max}/K_m (ml · mg^{-1} · min^{-1})
Taurocholate	1800	10	180
LTC_4	136	0.32	425
Daunorubicin	280	20	14

The kinetic constants were derived from double reciprocal plots according to Lineweaver and Burk. Transport studies were performed as described in section 2. Values are from quadruplicate determinations with all standard deviations at less than 15% of the mean.

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