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Evidence for multiple glucuronide transporters in rat liver microsomes

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Abstract

The transport of glucuronides across the endoplasmic reticulum membrane is an important step in the overall process of biotransformation, although the mechanism remains unclear and the participating transporters are unidentified. Using a rapid filtration assay in combination with liquid chromatography–mass spectrometry, we measured the transport of a variety of β-D-glucuronides in rat liver microsomes and investigated the substrate specificity of the participating transporter(s) by inhibition studies. Time-dependent and bi-directional transport of phenolphthalein glucuronide was detected and the kinetic parameters for transport were determined. The K_m and V_{max} values of high affinity transport were 26 μM and 3.9 nmol/min/mg protein, respectively. Phenolphthalein glucuronide transport was inhibited by 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid and *N*-ethylmaleimide. Transport inhibition studies revealed competition between three glucuronides: phenolphthalein glucuronide, estradiol 17-glucuronide and naphthol AS–BI glucuronide indicating that they share a common transporter in the endoplasmic reticulum membrane. Their transport was inhibited by phenolphthalein, but was not affected by *p*-nitrophenyl glucuronide, naphthyl glucuronide or D-glucuronate. Morphine 3-glucuronide transport was not inhibited by any of the latter four compounds or by phenolphthalein glucuronide. This novel experimental approach has produced data consistent with the presence of multiple (at least three) transporters catalyzing the transport of glucuronides through the endoplasmic reticulum membrane. These data also indicate that the size and/or shape of the aglycone rather than the glucuronic acid moiety per se is an important determinant of transporter specificity.

Keywords: Glucuronide; Endoplasmic reticulum; Transport; Kinetics; Inhibition; Rat liver

1. Introduction

Glucuronidation is quantitatively the most important phase II biotransformation reaction in the liver. It plays a major role in the inactivation and elimination of endobiotics, such as bilirubin and steroid hormones and of a wide variety of xenobiotics, including many drugs [1]. These molecules (aglycones) are imported into the endoplasmic reticulum (ER) as substrates for the UDP-glucuronosyltransferases (UGTs), which catalyze the conjugation of glucuronic acid to nucleophilic functional groups (e.g., hydroxyl, carboxyl, amino or thiol groups) [2]. The conjugated products of glucuronidation exit the ER lumen by an unknown mechanism and they are finally transported from the cytosol into the bile or into the blood by conjugate export pumps. Glucuronide transport across the plasma membrane is a relatively well characterized step of biotransformation, and several members of the multidrug resistance associated protein family (MRP1, 2 and 3) [3,4] and the organic anion transport proteins (OATP2, 4 and 8) [5–7] have been shown to be involved.

The UGTs are integral ER membrane proteins, and the results of structural and functional studies indicate that their active centers are localized in the lumen [8]. It is widely accepted that the latency of UGTs, that is the substantial difference between their activity in intact and permeabilized microsomes [9], is a consequence of this topology. The substrates (aglycone and UDP-glucuronic

Abbreviations: ER, endoplasmic reticulum; UGT, UDP-glucuronosyltransferase; MRP, multidrug-resistance-associated protein; OATP, organic anion transport protein; LC, liquid chromatography; MS, mass spectrometry; PhG, phenolphthalein β-D-glucuronide; NEM, *N*-ethylmaleimide; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; MOPS, 4-morpholinepropanesulfonic acid

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acid) must enter and the product (glucuronide) must exit the lumen of the ER through the membrane. Glucuronides are amphipathic compounds; the polarity and hydrophilicity of the aglycone are generally increased by conjugation. Moreover, the carboxyl group of the glucuronic acid moiety is dissociated at the pH of the ER lumen or the cytosol giving the compound a negative charge. Biological lipid bilayers are essentially impermeable to such bulky polar and charged molecules, which suggests that the glucuronide efflux is a protein mediated transport process.

Although it is evidently a crucial step in the overall process of glucuronide excretion [10,11], very little is known about glucuronide transport in the ER, and none of the transporter proteins involved has been identified. The presence of multiple ER glucuronide transporters is supported by the observation that bilirubin glucuronide transport was defective while 1-naphthyl glucuronide transport was normal in microsomes prepared from a hyperbilirubineamic infant [12]. The possible role of selective ER transporters in glucuronide sorting for excretion via bile canalicular or basolateral membranes has also been suggested [12].

The activity of a multifunctional ER membrane transporter, which translocates estradiol 17-glucuronide, has been recently reported [13]. *cis* inhibition studies showed that the as yet unidentified protein also transports estradiol 3-glucuronide, phenolphthalein glucuronide, sulfoconjugates and some other anionic compounds but not *p*-nitrophenyl glucuronide, *p*-acetamidophenyl glucuronide or glucuronate. On one hand, these findings indicate that the broad substrate specificity of this glucuronide transporter is not driven by the glucuronic acid moiety. On the other hand, these observations further support the presence of multiple glucuronide transporters of different specificity in the ER membrane.

The aim of the present study was to investigate the substrate specificity of the glucuronide transporter(s) in the hepatic ER membrane. Using a sensitive and precise LC/MS detection method, coupled to a rapid filtration assay, we investigated the transport of selected glucuronides of different size and shape in rat liver microsomes. Our results provide strong evidence for the presence of at least three different glucuronide transporters in the ER membrane with partly overlapping substrate specificity.

2. Materials and methods

2.1. Materials

Phenolphthalein β-D-glucuronide (PhG), *p*-nitrophenyl β-D-glucuronide, α-naphthyl β-D-glucuronide, 17-β-estradiol 17-(β-D-glucuronide), α-naphthol AS–BI β-D-glucuronide, morphine 3-(β-D-glucuronide), D-saccharic 1,4-lactone monohydrate (saccharolactone), UDP-glucuronic acid, *p*-nitrophenol, alamethicin, flufenamic acid, *N*-ethylmaleimide (NEM), 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) and 4-morpholinepropanesulfonic acid (MOPS) were purchased from Sigma Chemical Co. [Estradiol-6,7-^{3}H(N)]-estradiol 17-β-D-glucuronide (30–60 Ci/mmol) ([^{3}H]$E_2$17G) was obtained from Perkin-Elmer Life Sciences. All other reagents were of analytical grade.

2.2. Preparation of rat liver microsomal vesicles

Microsomes were prepared from livers of overnight fasted male Wistar rats (180–230 g), using fractional centrifugation [14]. The ER vesicles were washed and resuspended in MOPS–KCl buffer (100 mM KCl, 20 mM NaCl, 3 mM $MgCl_2$, 20 mM MOPS; pH 7.0) then immediately frozen in liquid nitrogen and kept in liquid nitrogen until use (within 6 months). The protein concentration in microsomal samples was determined using the method of Lowry et al. [15] with bovine serum albumin as a standard.

Purity of the microsomes was assessed by a marker-enzyme analysis [16]. Activities of glucose 6-phosphatase, cytochrome c oxidase, and 5′-nucleotidase as markers for endoplasmic reticulum, mitochondria, and plasma membrane, respectively, were determined in total tissue homogenate and in the microsomal fraction and the percentage of recovery was calculated. The results (glucose 6-phosphatase: 19.2 ± 1.8%; cytochrome c oxidase: 0.9 ± 0.1%; 5′-nucleotidase: 6.7 ± 0.6%) indicated an extent of contamination with mitochondria and plasma membrane similar to those usually reported for purified total microsomes [17–19].

The integrity of the microsomal membranes was assessed using the mannose-6-phosphatase assay [20], which showed a latency greater than 95%. Vesicle integrity was also confirmed by determination of the latency of *p*-nitrophenol glucuronidation in the microsomes. The intact microsomal vesicles (0.25 mg protein/ml) were incubated in MOPS–KCl buffer containing 0.3 mM *p*-nitrophenol and 4 mM UDP-glucuronic acid at 37 °C. The permeabilized samples containing the pore-forming antibiotic alamethicin (0.1 mg/mg protein) [21] were run in parallel. *p*-Nitrophenol consumption was continuously detected with a microplate reader (SpectraMax 190, Molecular Devices) at 405 nm wavelength. The activity of the intact microsomes was consistently 5–7% of the activity measured in the permeabilised microsomes, which corresponds to a latency of 94%, correlating closely with the mannose-6-phosphatase assay data.

2.3. Glucuronide transport measurements

The influx or efflux of glucuronides was determined using a rapid filtration method at 20 °C. In case of influx measurements, the microsomal fractions were resuspended (at 2 mg/ml protein concentration) and preincubated for 1 h in MOPS–KCl buffer containing 10 mM saccharolactone at 20 °C to inhibit microsomal β-glucuronidase [22].

When phenolphthalein glucuronide efflux was measured the microsomes were treated similarly, but the suspension (10 mg protein/ml) also contained phenolphthalein glucuronide at 50 μM final concentration. Uptake was initiated by the addition of the appropriate glucuronide dissolved in the same buffer in equal volume so that the final protein concentration was 1 mg/ml and the glucuronide concentration was 50 μM unless otherwise indicated. Release was started by a 20-fold dilution of the preloaded microsomes with MOPS–KCl buffer. In either case, samples containing 100 μg microsomal protein were withdrawn from the incubation mixtures at the indicated times, filtered immediately through cellulose acetate/nitrate filter membranes (MF-Millipore, pore size 0.45 μm) and washed quickly on the filter with 3 ml of ice-cold MOPS–KCl buffer.

The membrane bound fraction of glucuronides was measured in each experiment by performing the same procedure with permeabilized microsomes, which had been pretreated with the pore-forming reagent alamethicin (0.1 mg/mg microsomal protein). The intraluminal glucuronide content was calculated as the alamethicin-releasable content of the vesicles.

2.4. Glucuronide extraction, separation and measurement

The filter-retained glucuronides were measured by LC/MS without radioactive labeling. The glucuronides were extracted from the filters with 50% acetonitrile, separated by HPLC (Aligent HP1100 LC system, Aligent technologies) with detection via direct infusion into a mass spectrometer (Micromass LC Quattro, Micromass). Compounds were eluted from an ODS2 column with an increasing gradient of acetonitrile containing 0.1% formic acid. MS analysis was performed with electrospray using a multiple reaction monitoring. Details of these analyses will be published elsewhere. The extraction efficiency was above 95% in case of phenolphthalein glucuronide or morphine glucuronide and it was consistently 75 or 45% in case of estradiol glucuronide or naphthol AS–BI glucuronide, respectively at all concentrations tested. The microsome-associated glucuronide content therefore could be reliably determined with this method.

Some experiments were also carried out using radiolabeled tracer ([estradiol-6,7-^{3}H(N)]-estradiol 17-β-D-glucuronide; 10–15 mCi/mmol) together with the unlabeled glucuronide. In these experiments, the filters were dissolved in 5 ml scintillation liquid (Filter-Count LSC, Packard Bioscience) and the radioactivity in the samples was quantified in a liquid scintillation counter (Beckman-LS6500, Beckman).

2.5. Effect of transport inhibitors on phenolphthalein glucuronide transport

The effect of the anion transport inhibitors DIDS and flufenamate and the protein thiol reagent NEM on the PhG uptake was also investigated. The microsomes (2 mg protein/ml) were pre-incubated with each inhibitor for 20 min at 20 °C. They were then diluted two-fold and incubated with 50 μM PhG for 20 s at 20 °C. The initial rate of the influx was determined with rapid filtration as discussed above. The extraction efficiency, separation and detection of PhG were not influenced by the inhibitors.

2.6. Studies on the cis inhibition of glucuronide transport with selected glucuronides, glucuronate and aglycones

The possible competitive inhibition of glucuronide transport was assessed by measuring the initial rate of the transport at 20 s and 20 °C in the presence of another glucuronide (50 or 100 μM), glucuronate (100 μM) or a selected aglycone (50 μM). The extraction efficiency, separation and detection of the substrate glucuronides were tested in solutions containing each of the putative inhibitors and we found no interference.

2.7. Analysis of the stability of glucuronides in the conditions of the experiments

The molecular integrity of the glucuronides was continuously controlled during the transport experiments. The samples were assessed using LC/MS to detect the incidental appearance of glucuronate or aglycone, which would have indicated degradation. At the end of each experiment, total samples were taken and analyzed the same way. The initial and final amounts of glucuronides were also compared to observe any loss. The degradation of all glucuronides used in this work remained under 0.5%.

2.8. Statistics

Experiments were performed at least in triplicate, with each of the values of a single set of experiments corresponding to the mean of a minimum of 2–3 determinations ± S.E. Mean values were compared using Student's *t*-test.

3. Results

3.1. Phenolphthalein glucuronide uptake and release in rat liver microsomal vesicles

The time courses of inward and outward transport of PhG through the microsomal membrane were first compared to establish suitable conditions for subsequent experiments. Incubation of the microsomes with 50 μM PhG resulted in a time-dependent increase in the amount of the vesicle-associated substrate, which was radically reduced by pretreatment of the microsomes with the pore-forming antibiotic alamethicin (Fig. 1A). The intravesicular PhG content and thus all the activities were calculated from the difference between the values mea-

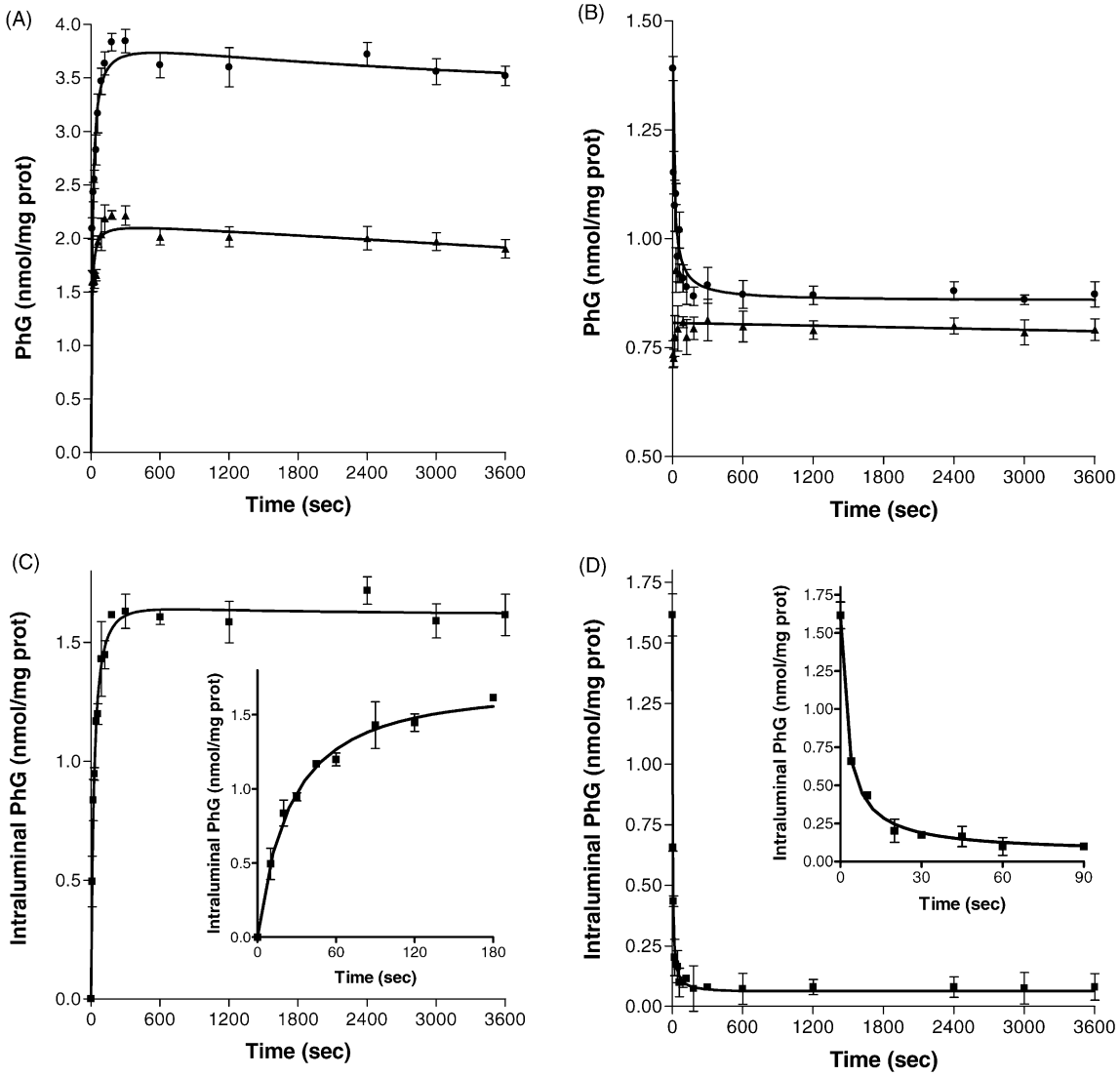

Fig. 1. Time-dependence of phenolphthalein β-D-glucuronide transport in rat liver microsomes. Uptake measurement was started by adding 50 μM PhG to rat liver microsomes (1 mg protein/ml). (A) The vesicle-associated glucuronide was measured in intact (●) and permeabilized (▲) microsomes at the indicated times by the rapid filtration method and LC/MS detection as described in materials and methods. (B) The intraluminal content was calculated from the difference. (C) Vesicle-associated PhG was also measured in intact (●) and permeabilized (▲) samples in release assays initiated by 20-fold dilution of the microsomes preloaded with 50 μM PhG. (D) The amount of intraluminal glucuronide was calculated by subtraction. Data are mean ± S.E.M. from three experiments.

sured in intact and permeabilized microsomes. The luminal accumulation of PhG was linear for the first 30 s and reached the equilibrium in about 5 min (Fig. 1B). The steady state was unchanged for at least 60 min. According to these observations, 20 s incubations were used to measure the initial transport activities in the subsequent experiments.

The microsomes were pre-loaded with 50 μM PhG at 10 mg/ml protein concentration by incubation at 20 °C for 60 min for the efflux measurements. The experiment was then started with a 20-fold dilution of the samples in order to create an outward gradient. The vesicle-associated glucuronide was measured in intact and permeabilized samples (Fig. 1C) and the intraluminal content was calculated by subtraction. Since the real initial (0 s) value is technically impossible to measure we plotted the release time course starting from the steady state value determined in the uptake measurements (Fig. 1D). The transport of PhG proved to be bi-directional; the intraluminal glucuronide content decreased linearly in the first 10 s and long-term equilibrium (measured for 60 min) was reached in approximately 2 min. Time-dependence of inward and outward transport was similar (initial rates were 2.54 ± 0.15 and 7.2 ± 0.77 nmol/min/mg protein, respectively). The apparently higher speed of efflux can be attributed to the inevitable loss of intravesicular glucuronide when the samples are washed on the filter.

The possible ATP-dependence of PhG transport was also investigated. In agreement with previous observations [13], ATP did not affect the rate of PhG transport across the ER membrane. When the glucuronide was added to the microsomes together with ATP (100 μM), the initial rate of

uptake (2.57 ± 0.28 nmol/min/mg protein) did not differ from the control.

3.2. Kinetic parameters of phenolphthalein glucuronide transport

The concentration-dependence of the initial rate of PhG uptake was studied using a concentration range between 10 μM and 10 mM. The Eadie–Hofstee plot clearly indicates the presence of two different transport activities: one with high affinity but relatively low capacity (K_m = 26 μM; V_{max} = 3.9 nmol/min/mg protein) and an other one with much lower affinity (K_m = 2.0 mM; V_{max} = 24.5 nmol/min/mg protein) (Fig. 2). These parameters make it likely that the high-affinity transporter plays the major role in vivo therefore we used 50 μM substrate concentrations in subsequent experiments to avoid any significant interference of the other activity.

3.3. Influence of transport inhibitors on phenolphthalein glucuronide transport

In order to collect further evidence for the participation of an anion transporter in the process, the effect of NEM, DIDS and flufenamate on PhG uptake was also evaluated. NEM, a thiol-alkylating reagent which has been shown to inhibit various transporters, caused about 30% inhibition while DIDS, a prototypic anion transport inhibitor, decreased the initial rate of transport by more than 60% (Fig. 3). Preincubation of the microsomes with flufenamate failed to influence PhG transport significantly (Fig. 3).

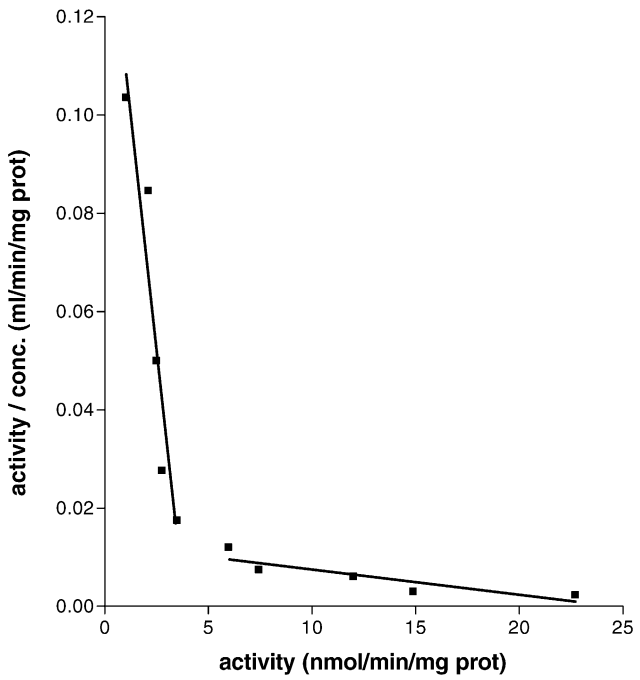

Fig. 2. Kinetic analysis of phenolphthalein β-D-glucuronide uptake in rat liver microsomes. PhG influx was measured at various concentrations (between 10 μM and 10 mM) in rat liver microsomes (1 mg protein/ml). The intraluminal glucuronide content was determined after 20 s incubation with the glucuronide at 20 °C. The initial rate was calculated at each concentration and the means of three experiments were plotted in Eadie–Hofstee format.

3.4. Inhibition of PhG transport by glucuronides, glucuronate and aglycones

The initial rate of transport in the presence of various glucuronides or glucuronate on the *cis* side of the membrane was measured in order to study the substrate specificity of the phenolphthalein glucuronide transporter. A significant and concentration-dependent inhibition was observed when estradiol 17-glucuronide was added to the incubation. Naphthol AS–BI glucuronide was found to be even more effective, resulting in 75% inhibition at 100 μM. Glucuronate, the smaller *p*-nitrophenyl glucuronide and naphthyl glucuronide and the larger morphine glucuronide, however proved to be ineffective at concentrations up to 100 μM (Fig. 4).

The type of inhibition caused by naphthol AS–BI glucuronide was studied by comparing the kinetics of PhG transport in the presence or absence of the inhibitor. The initial rate of transport was determined at various PhG concentrations in a range from 12.5 to 200 μM. The Lineweaver–Burk plot (Fig. 5) showed that the V_{max} was virtually unaltered while the K_m increased significantly (53 ± 4.9 μM versus 26 ± 3.2 μM) in the presence of naphthol AS–BI glucuronide, which indicated that the two compounds compete for the same transporter. When the effect of some relevant aglycones on the transport of phenolphthalein glucuronide was studied, a significant inhibition with phenolphthalein was observed (about 50% at 50 μM) (Fig. 4). The other aglycones investigated (morphine, *p*-nitrophenol, estradiol and naphthol) did not influence the transport significantly (data not shown).

The effect of all these glucuronides and aglycones on the outward transport of PhG was also investigated and similar results were obtained (data not shown), which suggested that the transport was mediated by the same transporter(s) in both directions.

The time course of PhG efflux was measured using a wash-buffer supplemented with 60 μM phenolphthalein in order to demonstrate that the higher rate of PhG efflux is a consequence of the loss of intraluminal glucuronide during filtration and washing. Compared to the previously shown results, the release appeared to be slower and its time course better approximated that of the uptake (Fig. 6).

3.5. Effect of glucuronides and aglycones on estradiol 17-β-D-glucuronide and naphthol AS–BI glucuronide transport

In order to confirm that PhG, estradiol 17-glucuronide and naphthol AS–BI glucuronide share a common transporter that is sensitive to phenolphthalein we investigated

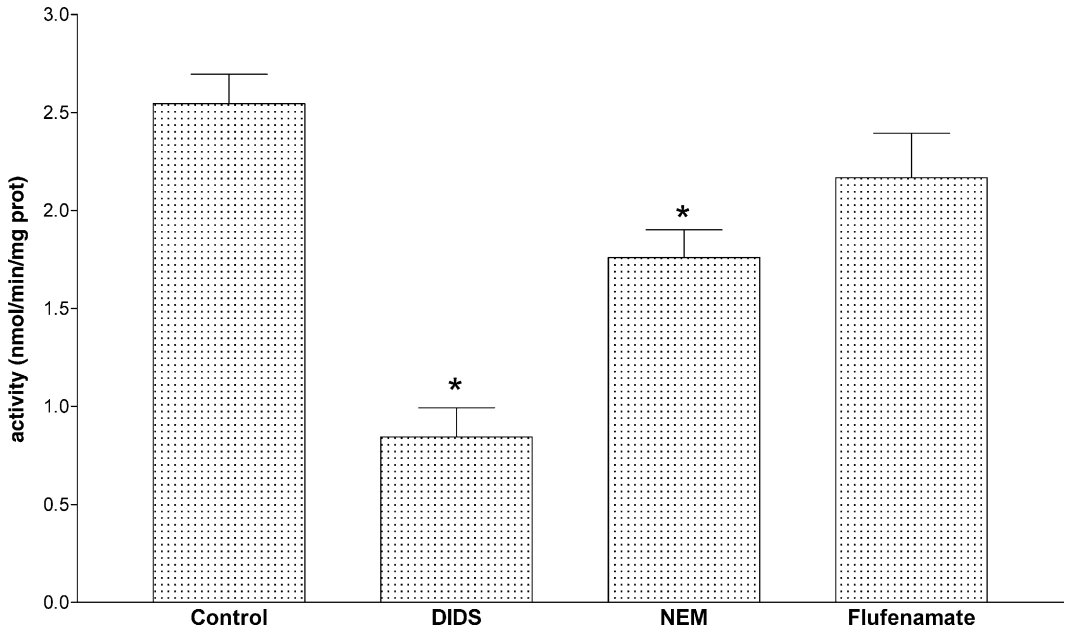

Fig. 3. Inhibition of phenolphthalein β-D-glucuronide transporter. Rat liver microsomes (2 mg protein/ml) were pretreated with the indicated agents (1 mM DIDS, 5 mM NEM or 0.1 mM flufenamate) for 20 min at 20 °C. Then they were diluted two-fold and incubated with 50 μM PhG at 20 °C for 20 s. The initial rate of the inward transport was measured with rapid filtration. Data are mean ± S.E.M. from three experiments. $^{*}P < 0.003$ compared with control.

the effect of the selected glucuronides and aglycones on the uptake of estradiol 17-glucuronide and naphthol AS–BI glucuronide in rat liver microsomes. The time course of estradiol 17-glucuronide (50 μM) influx was determined and – in agreement with previous observations [13] – a linear increase in intravesicular glucuronide content within the first 35–40 s was observed. The microsomes therefore, were incubated for 20 s in the presence of 50 μM estradiol 17-glucuronide with or without selected glucuronides (100 μM), aglycones (50 μM) or glucuronate (100 μM)

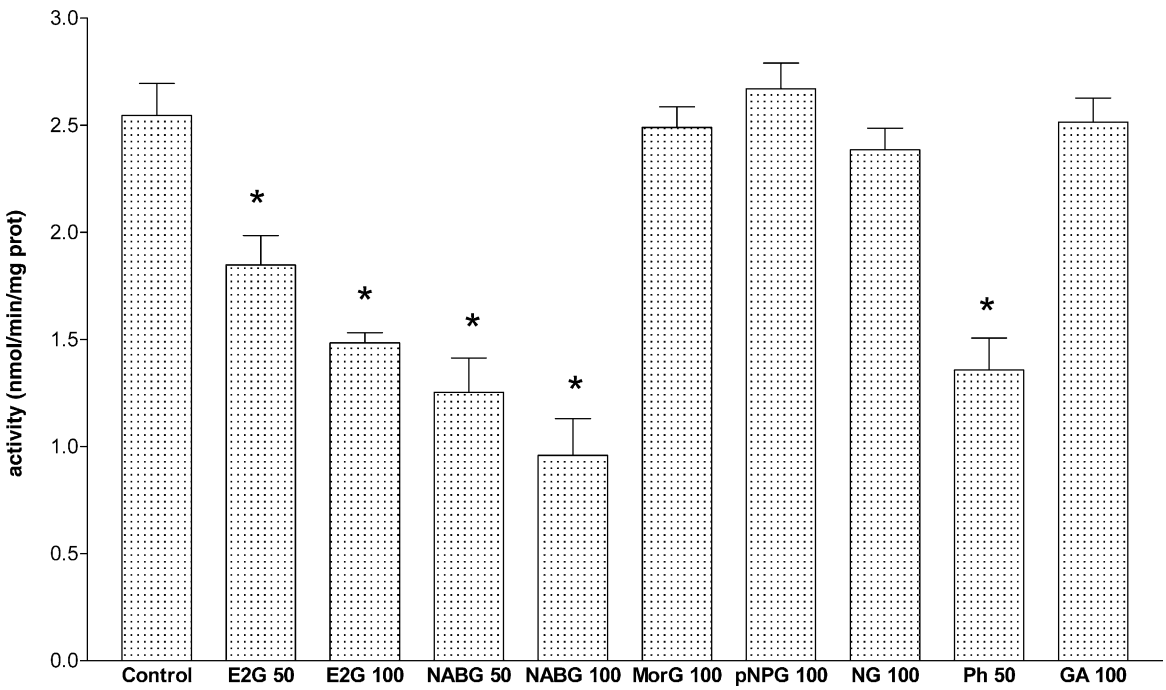

Fig. 4. Effect of putative competitors on phenolphthalein β-D-glucuronide transport. Rat liver microsomes (1 mg protein/ml) were incubated at 20 °C for 20 s with 50 μM PhG and the intravesicular glucuronide content was determined with rapid filtration to calculate the initial rate of the inward transport. The indicated putative competitor was added together with PhG to the vesicles at 50 or 100 μM concentration as the number shows. E2G: estradiol 17-glucuronide; NABG: naphthol AS–BI glucuronide; MorG: morphine 3-glucuronide; pNPG: *p*-nitrophenyl glucuronide; NG: naphthyl glucuronide; Ph: phenolphthalein; GA: glucuronate. Data are mean ± S.E.M. from three experiments. $^{*}P < 0.003$ compared with control.

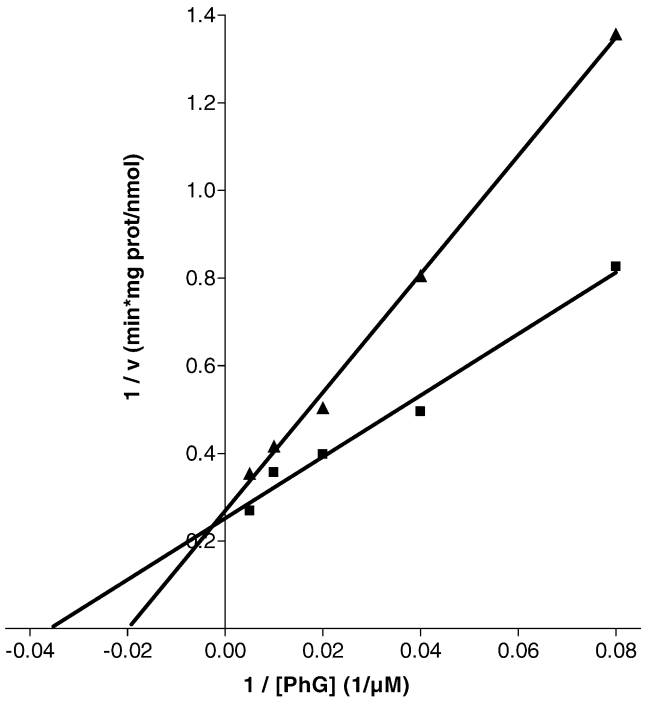

Fig. 5. Naphthol AS–BI β-D-glucuronide inhibits phenolphthalein β-D-glucuronide transport competitively. PhG influx was measured at various PhG concentrations (from 10 to 200 μM) at 20 °C in rat liver microsomes (1 mg protein/ml). The microsomes were incubated in the presence (▲) or absence (■) of 30 μM naphthol AS–BI glucuronide. The initial rate was determined at each PhG concentration from the intraluminal glucuronide content after 20 s incubation and the means of three experiments were plotted in Lineweaver–Burk format.

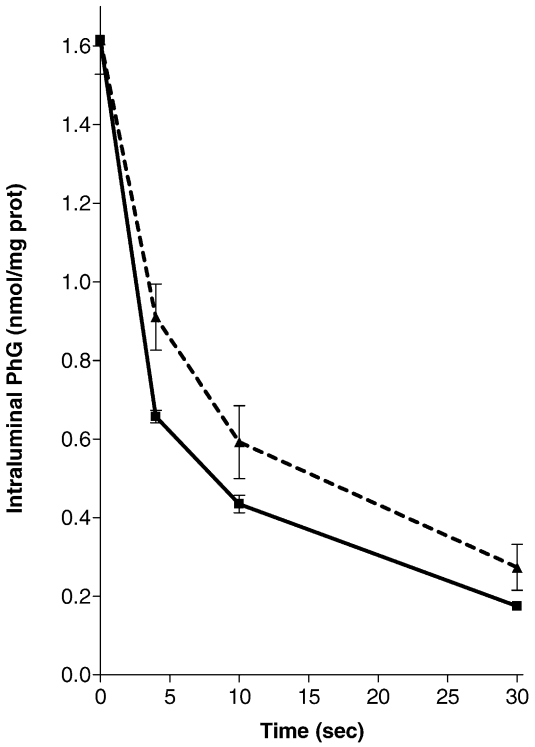

Fig. 6. Phenolphthalein inhibits phenolphthalein β-D-glucuronide efflux in rat liver microsomes. The time course of PhG release was determined in rat liver microsomes. The vesicles were preloaded with 50 μM PhG then diluted 20-fold to create an outward gradient. The intraluminal glucuronide content was determined with the rapid filtration assay. The vesicles were washed either with ice-cold MOPS–KCl buffer (continuous line) as described in Section 2 or with the same buffer supplemented with 60 μM phenolphthalein (broken line). Values are mean ± S.E.M. from three experiments.

to determine the initial rate of the transport. PhG caused about 50% inhibition while *p*-nitrophenyl glucuronide and glucuronate failed to influence the transport (Table 1), as reported previously [13]. Naphthyl glucuronide and morphine 3-glucuronide were also ineffective, however substantial inhibition was observed when naphthol AS–BI glucuronide or phenolphthalein was added (Table 1). The corresponding aglycone, estradiol had a smaller but significant effect (initial rate was 1.25 ± 0.11 nmol/min/mg protein vs. 1.58 ± 0.03 nmol/min/mg protein, $n = 3$, $P < 0.05$). All these results including the time course were confirmed using ^{3}H-labeled estradiol 17-glucuronide.

The uptake of 50 μM naphthol AS–BI glucuronide was linear for about 20 s (data not shown), therefore the initial

Table 1
Inhibition of estradiol 17-β-D-glucuronide, naphthol AS–BI β-D-glucuronide and morphine 3-β-D-glucuronide transport by putative competitors in rat liver microsomes

Competitor	% of control[a]		
	Estradiol 17-β-glucuronide uptake	Naphthol AS–BI glucuronide uptake	Morphine 3-β-glucuronide uptake
None	100.0 ± 1.9	100.0 ± 3.3	100.0 ± 10.6
p-Nitrophenyl β-glucuronide	100.5 ± 5.1	99.1 ± 4.4	103.7 ± 6.0
Naphthyl β-glucuronide	98.7 ± 6.2	97.0 ± 6.8	99.8 ± 7.1
Phenolphthalein β-glucuronide	50.5 ± 2.5*	72.3 ± 4.1*	96.1 ± 9.8
Estradiol 17-β-glucuronide	–	81.5 ± 2.7*	18.5 ± 3.6*
Naphthol AS–BI β-glucuronide	48.6 ± 2.2*	–	52.3 ± 5.1*
Morphine 3-β-glucuronide	98.1 ± 4.4	96.4 ± 5.5	–
Phenolphthalein	45.1 ± 2.9*	62.3 ± 7.6*	100.6 ± 13.2
Glucuronate	102.5 ± 3.4	101.2 ± 4.0	95.9 ± 8.8

Data are mean ± S.E.M. from three experiments.

[a] Control values: estradiol 17-glucuronide: 1.58 ± 0.03 nmol/min/mg protein, naphthol AS–BI glucuronide: 27.07 ± 0.89 nmol/min/mg protein, morphine 3-glucuronide: 23.5 ± 2.5 pmol/min/mg protein

* $P < 0.005$ compared with control.

rate was determined in 10 s incubations. PhG, estradiol 17-glucuronide and phenolphthalein inhibited the transport significantly (by 30, 20, and 40%, respectively) while *p*-nitrophenyl glucuronide, naphthyl glucuronide and glucuronate did not (Table 1).

3.6. Effect of putative competitors on morphine 3-β-D-glucuronide transport

The previous results showed that the smaller *p*-nitrophenyl glucuronide and naphthyl glucuronide as well as the bulkier morphine 3-glucuronide did not influence the transport of PhG, naphthol AS–BI glucuronide and estradiol 17-glucuronide. We therefore investigated whether PhG, naphthol AS–BI glucuronide and estradiol 17-glucuronide could inhibit the transport of the other three compounds. First we tried to determine the time courses of the influx of *p*-nitrophenyl glucuronide, naphthyl glucuronide and morphine 3-glucuronide in rat liver microsomes. When the microsomes (1 mg protein/ml) were incubated with 50 μM *p*-nitrophenyl glucuronide, no accumulation (above the membrane binding) was observed using the rapid filtration method. The permeability of the ER membrane is much higher to *p*-nitrophenyl glucuronide than to PhG as previously shown [11]. This high permeability can cause the complete loss of intraluminal *p*-nitrophenyl glucuronide during filtration and washing, and explains the apparent lack of glucuronide accumulation. Similarly, the transport of 50 μM naphthyl glucuronide was so rapid that its accumulation could not be detected with rapid filtration i.e. the microsomes reached steady state within the first 10 s.

The time-dependence of morphine 3-glucuronide uptake could be detected with the rapid filtration method. The transport of this bulky glucuronide (at a concentration of 50 μM, at 20 °C) was much slower (23.5 ± 2.5 pmol/min/mg protein) than that of PhG. The accumulation was linear for about 5 min and it took more than 30 min to reach the steady state. In subsequent inhibition experiments therefore we determined the initial rate of transport after 1 min incubations.

Rat liver microsomes were incubated with 50 μM morphine 3-glucuronide at 20 °C for 1 min in the presence of various potential competitors: glucuronate (100 μM), glucuronides (100 μM) or aglycones (50 μM). PhG failed to inhibit morphine 3-glucuronide transport (Table 1) in accordance with our previous observations. On the other hand, both estradiol 17-glucuronide and naphthol AS–BI glucuronide reduced the rate of the uptake (Table 1). Glucuronate, *p*-nitrophenyl glucuronide and naphthyl glucuronide, which did not inhibit the transport of any of the glucuronides investigated, were again ineffective (Table 1). Phenolphthalein, the aglycone that substantially inhibited the transport of PhG and of estradiol 17-glucuronide, did not have a similar effect on morphine 3-glucuronide uptake (Table 1).

4. Discussion

Biotransformation was classically divided into two phases: (i) first phase: the preparation (mostly hydroxylation) of endobiotics or xenobiotics for (ii) second phase: their conjugation (e.g. glucuronidation). Recently, the transport of substrates and conjugated products across cell membranes has become of increasing interest, and is now recognized as a key component of biotransformation reactions. These processes are often referred to as zero phase (uptake of substrate) and third phase (export of conjugate), respectively. Very little attention has been paid, however, to the transport processes across the ER membrane although they are important determinants of the intraluminal UGT activities and glucuronidation cannot be properly studied without elucidating them. The fact that ER transport is generally poorly investigated is probably due to technical difficulties. Current analytical techniques for studying transport across the microsomal membrane are limited and rely on the availability of radiolabeled substrates. Using a novel application of LC/MS detection, we measured the ER transport of glucuronides of different size and shape and investigated the ligand-specificity of their yet unidentified transporter(s).

Characterization of the microsomal transport of PhG revealed that it was protein-mediated (saturable, sensitive to transport-inhibitors and susceptible to competitive inhibition), ATP-independent and bi-directional. The transport of estradiol 17-glucuronide has been shown to have similar characteristics [13], suggesting that the general mechanism of glucuronide transport across the ER membrane is facilitated diffusion. The glucuronides to be eliminated are synthesized by UGTs in the ER lumen. It is not clear whether glucuronides entering hepatocytes are normally imported into the ER for hydrolysis by the microsomal β-glucuronidase [12] in the lumen. Hence the major physiological function of glucuronide transporters in the ER membrane is believed to be the export of glucuronide conjugates into the cytosol. However, for practical reasons, we reduced the number of efflux measurements and studied the influx in most experiments. It is generally accepted that facilitated diffusion can be investigated in both directions regardless of the theoretically preferential orientation [13,23–26].

Transport of estradiol 17-glucuronide across the plasma membrane by MRPs, MDR1 and OATPs has been reported [4,27–30]. Moreover, it has been demonstrated that the transport by MRP2 is competitively inhibited by phenolphthalein glucuronide but not by naphthyl glucuronide or *p*-nitrophenyl glucuronide [31], which is very similar to our observations. However, the plasma membrane pumps transport estradiol glucuronide in an ATP-dependent manner and OATPs are modulated by GSH [32]. The previously reported ATP- and GSH-independence of estradiol 17-glucuronide transport in the ER membrane [13], therefore strongly suggests that the translocation mechanism in

this membrane is distinct from those in the plasma membrane. The possible role of MRPs and MDRs in the transport of glucuronides across the ER membrane is further contradicted by their orientations. These pumps translocate their substrates from the cytosol to the extracellular space, thus in the ER they would pump glucuronides into the lumen, where they are synthesized and should be released from.

The results presented suggest an inverse correlation between the size of the compounds we studied and the rate of their transport across the ER membrane. *p*-Nitrophenyl glucuronide crossed the ER membrane so quickly that the rapid filtration method could not be employed, and naphthyl glucuronide transport reached steady state within a few seconds. Transport of naphthol AS–BI glucuronide and PhG was fast but measurable with rapid filtration, whereas estradiol 17-glucuronide transport was slower and the bulkiest compound, morphine 3-glucuronide, was transported remarkably slowly through the ER membrane. Despite their very rapid transport, *p*-nitrophenyl glucuronide and napthyl glucuronide did not affect the traffic of any of the other glucuronides investigated. This supports the idea that these small compounds are predominantly transported via a separate mechanism (transporter) in the ER, which does not participate significantly in the transport of larger glucuronides. The existence of a transporter specific to small glucuronides is further supported by the finding of Battaglia et al. [13] that *p*-acetamidophenyl β-glucuronide, another compound of very similar size to the glucuronides of *p*-nitrophenol and α-naphthol, also failed to inhibit estradiol 17-glucuronide transport in rat liver microsomes. Similarly morphine 3-glucuronide, having the bulkiest rigid non-planar structure among the compounds studied, did not interfere with the transport of other glucuronides and its transport was only inhibited by estradiol 17-glucuronide and naphthol AS–BI glucuronide, but not by PhG. It is therefore likely that the transporter of morphine 3-glucuronide predominantly accepts bulky substrates. Our observation that estradiol 17-glucuronide and naphthol AS–BI glucuronide significantly inhibited morphine 3-glucuronide transport but not vice versa can be explained by the different rate of flux for the various compounds. Morphine 3-glucuronide crossed the membrane very slowly; so the contribution of its transporter to the overall estradiol 17-glucuronide and naphthol AS–BI glucuronide transport might be negligible.

The reciprocal inhibition between PhG, estradiol 17-glucuronide and naphthol AS–BI glucuronide indicates that these three compounds share a common transporter in the ER membrane. It is also remarkable that phenolphthalein inhibited this transporter while it failed to interfere with morphine 3-glucuronide transport.

In conclusion, our results are consistent with the presence of multiple (at least three) glucuronide transporters of wide and partially overlapping substrate specificity in the hepatic ER membrane. Interestingly, the specificity pattern of the transporters shows similarities with that of the corresponding UGTs [33]. The glucuronate moiety seems to play a negligible role in interaction with the transporters as phenolphthalein inhibited the transport of some glucuronides while glucuronate was completely ineffective. Our findings show that the size and/or shape of the conjugated molecules are major determinants of specificity for these transporters while hydrophobicity appears less important. This observation is in accordance with the previously reported phenomenon that deficient transport of a large glucuronide (bilirubin glucuronide) was observed in the same ER sample as normal efflux of a smaller one (naphthyl glucuronide) [12]. The preferential targeting of bulky glucuronides towards the apical (canalicular) membrane and the smaller ones towards the basolateral membrane might be partly based on the size- and/or shape-selectivity of the transport of these conjugates in the ER membrane. Our results therefore have significant implications for understanding of the overall process of glucuronidation.

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