ELSEVIER

Biochimica et Biophysica Acta 1283 (1996) 223–231

BBA Biochimica et Biophysica Acta

Two kinetically-distinct components of UDP-glucuronic acid transport in rat liver endoplasmic reticulum

Eric Battaglia [a,b], Susan Nowell [a], Richard R. Drake [c], Magdalena Mizeracka [a], Carl L. Berg [d], Jacques Magdalou [b], Sylvie Fournel-Gigleux [b], John L. Gollan [d], Roger Lester [a], Anna Radominska [a,*]

[a] *Department of Internal Medicine, Division of Gastroenterology, University of Arkansas for Medical Sciences, 4301 W. Markham, Slot 567-1, Little Rock, AR 72204, USA*

[b] *Centre du Médicament, URA CNRS 597, Faculté des Sciences Pharmaceutiques et Biologiques, 54000 Nancy, France*

[c] *Department of Biochemistry and Molecular Biology, University of Arkansas for Medical Sciences, Little Rock, AR 72205, USA*

[d] *Gastroenterology Division, Brigham and Women's Hospital, Harvard Medical School and Harvard Digestive Disease Center, Boston, MA, USA*

Received 28 November 1995; revised 25 March 1996; accepted 13 May 1996

Abstract

Previous studies have documented the presence of protein-mediated transport of UDP-glucuronic acid (UDP-GlcUA) in rat liver endoplasmic reticulum (ER). Measurement of uptake at varying concentrations of high specific activity [β-^{32}P]UDP-GlcUA has revealed the presence of a two component UDP-GlcUA transporting system. Transport at low substrate concentrations occurred predominantly via a high affinity component (K_m = 1.6 μM), whereas a low affinity component (K_m = 38 μM) predominated at high substrate concentrations. The K_m for the high affinity system is in agreement with that previously published, while the low affinity component is a new finding. The uptake of UDP-GlcUA was temperature-sensitive, time dependent, and saturable for both components. The high affinity transport was affected by *trans*-stimulation and *cis*-inhibition by UDP-*N*-acetylglucosamine (UDP-GlcNAc); however, the same concentrations of UDP-GlcNAc had less effect on the low affinity system. In order to further study the two transport components, various inhibitors of anion transport carriers were tested. The high affinity component was strongly inhibited by 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS) and furosemide, while the low affinity system was less sensitive to these reagents. Dose-dependent inhibition by 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) was found for both transport systems. Probenecid was found to be a weak inhibitor of both components of the UDP-GlcUA uptake. Finally, the major metabolite of 3′-azido-3′-deoxythymidine, 3′-azido-3′-deoxythymidine monophosphate (AZTMP), was able to inhibit the uptake of UDP-GlcUA by both components. The results indicate the presence of two carrier-mediated UDP-glucuronic acid transporting components in rat liver ER.

Keywords: UDP-glucuronic acid; Endoplasmic reticulum; Transport; Glucuronidation; (Rat liver)

1. Introduction

UDP-Glucuronosyltransferases (UGTs) are a family of enzymes involved in the biotransformation of exogenous compounds, such as drugs and other xenobiotics, and endogenous compounds, including bilirubin, steroid hormones, bile acids and thyroid hormones [1,2]. Hepatic glucuronidation occurs primarily in the endoplasmic reticulum (ER) of the hepatocyte and results in the conversion of hydrophobic compounds to more hydrophilic species. Because UDP-glucuronic acid (UDP-GlcUA), the essential co-substrate of UGTs, is synthesized from UDP-Glc by UDP-glucose dehydrogenase in the cytosol of the hepatocyte and the active sites of the UGTs are lumenal in orientation [3–7], a mechanism presumably exists for the translocation of UDP-GlcUA from the cytosol to the ER lumen. A microsomal transport process for this highly hydrophilic substrate has been postulated for years [8–10]. Indeed, transport of UDP-GlcUA is an integral part of

Abbreviations: AZT, 3′-azido-3′-deoxythymidine; AZTMP, 3′-azido-3′-deoxythymidine monophosphate; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; ER, endoplasmic reticulum; SITS, 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid; UDP-GlcUA, UDP-glucuronic acid; UDP-Glc, UDP-glucose; UDP-Gal, UDP-galactose; UDP-GlcNAc, UDP-*N*-acetylglucosamine; UDP-Xyl, UDP-xylose; UGTs, UDP-glucuronosyltransferases

* Corresponding author. Fax: +1 501 6866248.

PII S0005-2736(96)00098-3

detoxification, a fact that often receives less attention than the aforementioned biotransformations.

The functional characterization of this UDP-GlcUA transporting system has been initiated recently by us and others. Bossyut et al. [11] employing dual-labeling and *trans*-stimulation experiments, demonstrated uptake of UDP-GlcUA into intact rat liver microsomal vesicles, consistent with bidirectional, carrier-mediated transport. This process was saturable, osmotically sensitive and inhibited by the protein-modifying reagent *N*-ethylmaleimide, supporting the involvement of a transmembrane protein carrier in this process. Our studies using a rapid filtration technique provided additional evidence for saturable transport of [^{14}C]UDP-GlcUA into an osmotically sensitive space of ER vesicles [12]. Moreover, uptake was found to be potently *cis*-inhibited and *trans*-stimulated by UDP-*N*-acetylglucosamine (UDP-GlcNAc). Additionally, GTP and Mg^{2+} were found to be putative in vivo effectors of UDP-GlcUA transport [12]. A mechanism of stimulation of UGT activity by UDP-GlcNAc has recently been proposed. This model describes the interactions of two transport pathways, i.e., UDP-GlcUA influx coupled to UDP-GlcNAc efflux, and UDP-GlcNAc influx coupled to UMP efflux, combined with intravesicular metabolism of UDP-GlcUA [13]. We have reported the photoincorporation of [β-^{32}P]5-azido-UDP-GlcUA ([β-^{32}P]5-N_3-UDP-GlcUA) into the active site of UGTs as an indicator of UDP-GlcUA uptake into ER microsomal vesicles, consistent with the involvement of a membrane carrier with a cytoplasmic face sensitive to partial tryptic digestion [14]. The amount of information provided in the above studies is substantial. However, except for the UDP-GlcUA uptake experiments performed with a very low concentration of substrate [12], the kinetic parameters of the uptake process could not be resolved. For this reason, we have re-examined the kinetics of the uptake at varying concentrations using [^{32}P]UDP-GlcUA.

In this report, we have further characterized the carrier-mediated process of UDP-GlcUA transport into rat liver ER vesicles using a rapid filtration technique. The availability of [β-^{32}P]UDP-GlcUA of high specific activity facilitated these studies of the initial rate of uptake over a wide range of substrate concentration. This approach led to the detection of two distinct components for the uptake of UDP-GlcUA in rat liver microsomes. We have characterized both components by performing *cis*-inhibition and *trans*-stimulation experiments with various UDP-sugars and evaluating the effects of different reversible and irreversible anion-transport inhibitors.

2. Materials and methods

2.1. Materials

Uridine diphosphate glucuronic acid, [glucuronyl-^{14}C(U)] (285.2 mCi/mmol) ([^{14}C]UDP-GlcUA) and uridine diphosphate galactose, [galactose-4,5^{3}H(U)] (30–50 Ci/mmol) ([^{3}H]UDP-galactose) were obtained from Dupont-NEN (Boston, MA). Filtron-X and Dimiscint were from National Diagnostics (Manville, NJ) and Soluene 350 from Packard (Downers Grove, IL). 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) and 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS) were from Molecular Probes (Eugene, OR). Uridine diphosphate glucuronic acid (UDP-GlcUA, triammonium salt), uridine diphosphate glucose (UDP-Glc, disodium salt), uridine diphosphate xylose (UDP-Xyl, sodium salt), uridine monophosphate (UMP, sodium salt) and UDP-*N*-acetylglucosamine (UDP-GlcNAc, sodium salt) were purchased from Sigma.

2.2. Synthesis of [β-^{32}P]UDP-GlcUA

[β-^{32}P]UDP-GlcUA was synthesized and purified as described for the synthesis of [β-^{32}P]5N_3UDP-GlcUA with UTP substituted for 5-azido-UTP [15]. [^{32}P]P_i was from ICN (Irvine, CA). Conversion of [β-^{32}P]UDP-Glc to [β-^{32}P]UDP-GlcUA ranged from 70 to 100% with a specific activity of 5–10 mCi/μmol.

2.3. Rat liver microsomal preparation

Intact liver microsomes from male rats (Sprague-Dawley, 220–250 g) were prepared as previously described [3], except that they were not further subfractionated into rough and smooth ER fractions. The microsomal fraction was resuspended in 0.25 M sucrose, 1 mM $MgCl_2$, 10 mM HEPES, pH 7.4. Aliquots of microsomes were rapidly frozen in liquid nitrogen and stored at −80°C. The protein concentration was determined by the Bradford method [16], using bovine serum albumin as the standard. The integrity of the microsomal vesicles was determined by a mannose-6-phosphatase latency assay [17], with the modifications of Blair and Burchell [18]. Latency of the vesicles was consistently found to be greater than 95%.

2.4. Marker enzyme activities

Mannose-6-phosphatase activity was determined in liver homogenates and microsomal fractions. The enrichment of the enzyme in microsomes over the homogenate was typically 3.4-fold, which is comparable to previously published values [13]. Na^+/K^+ ATPase activity was assayed as described in [19] to detect any contamination of microsomal preparations by plasma membranes. No detectable activity was found in the microsome preparations. The enzyme activity of UDP:galactose: ovomucoid galactosyltransferase, a marker of the Golgi membranes, was assessed according to [20] with the modification of [21], using [^{3}H]UDP-galactose. Liver homogenates and microsomal fractions were first treated with 4 mM 3-[(3-cholamidopropyl) dimethylammonio]-2-hydroxy-1-propane-

sulfonate (CHAPSO) on ice for 1 h [22] and enzyme activity was then determined.

2.5. Transport measurements

Uptake assays were performed as previously described [12] with the following modifications. The incubation medium contained rat liver microsomal vesicles (250 μg protein) with the appropriate concentrations of either [^{14}C]UDP-GlcUA or [β-^{32}P]UDP-GlcUA, as indicated in the figure legends, and uptake buffer (0.25 M sucrose, 1 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4), in a final volume of 100 μl. The specific activity was typically in the range of 0.45 μCi/nmol and 0.24 μCi/nmol, when the uptake of 5 μM and 75 μM [β-^{32}P]UDP-GlcUA, respectively, was examined. Uptake was initiated by the addition of rat liver microsomal vesicles to the uptake buffer, both of which had been preincubated at 25°C for 4 min. For experiments in which the initial rate of influx was measured, uptake was terminated after 15 s by the addition of 4 ml of an ice-cold 'stop' solution (0.25 M sucrose, 100 mM NaCl, 1 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4). The diluted microsomes were filtered immediately through a 0.22 μm cellulose filter (Micron Separations) presoaked in the same buffer containing 5 mM UDP-GlcUA using an ultrafiltration manifold (FH 225V, Hoefer Scientific Instruments) under vacuum, and the filter then washed with an additional 5 ml of 'stop' solution. Filters were dissolved in 10 ml of Filtron-X (National Diagnostics) and counted using a LKB 1214 Rackbeta liquid scintillation spectrometer. The values of vesicle-incorporated radioactivity were corrected for non-specific binding, determined by adding the incubation medium to the ice-cold stop solution and filtering as above.

2.6. Effect of osmolarity on [^{14}C]UDP-GlcUA uptake

Osmolarity studies were performed essentially as previously described [11]. Briefly, intravesicular volume was modified by incubation of microsomes (250 μg protein) for 15 min at 37°C with the non-permeant trisaccharide raffinose (0–150 mM) in 0.25 M sucrose, 1 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4. [^{14}C]UDP-GlcUA was then added to a final concentration of 5 or 75 μM and the incorporation of the radionuclide was determined, as described above, at equilibrium, previously established as 40 min at 37°C. The concentration of raffinose in the 'stop' solutions was identical to that used in the incubation medium. The osmolarity of the solutions was determined using a vapor pressure osmometer (5100C, Wescor).

2.7. Substrate specificity studies

cis-Inhibition of initial UDP-GlcUA uptake by various UDP-sugars (UDP-GlcUA, UDP-GlcNAc, UDP-Glc and UDP-Xyl) and UMP was investigated at concentrations of 0.1 and 1.0 mM. *trans*-Stimulation experiments were performed with these compounds by mechanically preloading the microsomal vesicles with the nucleotide sugars, as previously described [12], using the same concentrations used for the *cis*-inhibition studies (0.1 and 1.0 mM). The uptake for *trans*-stimulation experiments was evaluated following dilution of microsomes to 50 μg protein per assay to reduce the external UDP-sugar concentration and, thus, the *cis*-inhibition. The initial rates of UDP-GlcUA influx were corrected for possible *cis*-inhibition of uptake by the residual external UDP-sugar, which was determined as follows. Briefly, the uptake was measured on control microsomes (no preloading) at an external UDP-sugar concentration range of 1.4 to 14.0 μM, which corresponds to the external concentration that would exist during the *trans*-stimulation studies.

2.8. Inhibitor studies

The inhibitory potencies of furosemide, probenecid, AZT and AZTMP on the initial rate of uptake were evaluated by the external addition of these compounds to the microsomal vesicles. In control experiments, it was shown that 2% (v/v) dimethyl sulfoxide, used to solubilize furosemide and probenecid, did not affect the initial uptake rate. The dose-dependent, irreversible inhibition by SITS and DIDS was determined by incubating the microsomal vesicles (21.3 mg/ml protein) with these compounds for 10 min at 25°C in 0.25 M sucrose, 1 mM $MgCl_2$, 10 mM Hepes, pH 7.4. Incubations were then diluted 10 times in 0.25 M sucrose, 1 mM $MgCl_2$, 10 mM Tris-HCl pH 7.4 and initial uptake was measured. The degree of the inhibition is represented as a percentage of uptake activity compared to control experiments in which SITS or DIDS was omitted.

The possible loss of vesicle integrity in the presence of inhibitors used in these studies was checked using the mannose-6-phosphatase latency assay. Intact microsomes were treated with 1.2 mM SITS, 0.8 mM DIDS (the highest concentrations used for the determination of IC_{50} values; see Table 4), 1 mM probenecid or 2 mM furosemide and the mannose-6-phosphatase activity was measured and compared to that of untreated microsomes. No effect of the anion transport inhibitors was observed on the mannose-6-phosphatase activity either with intact microsomes or detergent-activated microsomes (not shown). Microsomal latency was in the range of 94.5–95.8%, either with control microsomes or microsomes pretreated with inhibitors, thus indicating no change in the integrity of the microsomal preparations in the presence of these compounds.

2.9. Statistics

Values in figures and tables are given as the mean ± S.E. of at least two independent experiments performed on two different microsomal preparations, unless otherwise indi-

cated. Testing for the significance of comparative data was done by the Student's t test with a confidence level of 95%.

3. Results

3.1. Kinetic evidence for the presence of a multi-component UDP-GlcUA transporting system in rat liver endoplasmic reticulum

Transport uptake at varying concentrations of high specific activity [β-^{32}P]UDP-GlcUA determined under initial rate conditions (15 s incubation) provided evidence for two UDP-GlcUA transporting components. As shown in Fig. 1, a double reciprocal plot of uptake at substrate concentrations ranging from 1–110 μM substrate was biphasic, and an Eadie-Hofstee plot of this uptake clearly indicated the presence of at least two components of UDP-GlcUA transport. One was characterized by a V_m of 30 ± 3 pmol · min^{-1} · mg^{-1} and a K_m of 1.6 ± 0.4 μM and is subsequently referred to throughout this study as the high affinity component of the UDP-GlcUA uptake into microsomal vesicles. The second saturable, low affinity component of this uptake was characterized by a V_m of 163 ± 23 pmol · min^{-1} · mg^{-1} and a K_m of 38 ± 7 μM. Consequently, two concentrations of radiolabeled UDP-GlcUA were chosen to study both the high affinity and the low affinity components of uptake (5 μM and 75 μM, respectively). The availability of high specific-activity [β-^{32}P]UDP-GlcUA allowed us to study higher substrate concentrations and do faster assays than with the commercially available ^{14}C analog which is expensive and has low specific activity. Both compounds behaved in the same manner in preliminary studies.

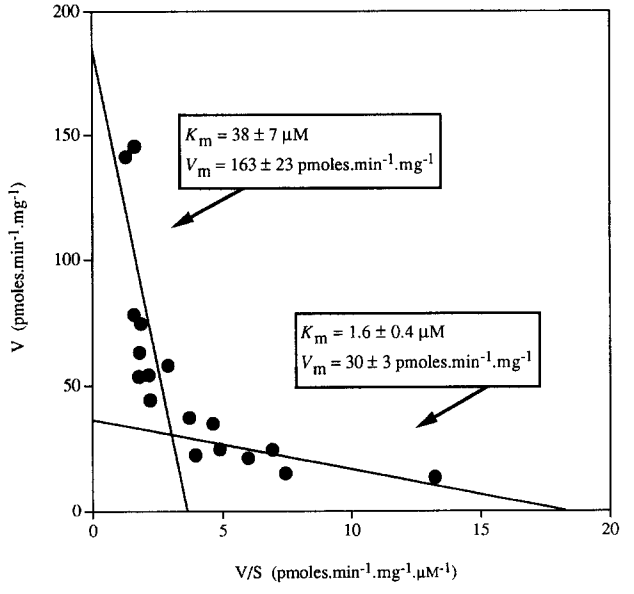

Fig. 1. Substrate concentration-dependence of the initial uptake rate of [β-^{32}P]UDP-GlcUA into ER vesicles. The initial uptake rate of radiolabeled UDP-GlcUA into ER vesicles (250 μg protein) was measured after 15-s incubation at 25°C in a concentration range of 1–110 μM substrate in 0.25 M sucrose, 1 mM $MgCl_2$, 10 mM Tris-HCl pH 7.4, by rapid filtration as described under Section 2. The figure is an Eadie-Hofstee plot of the data for one representative experiment while V_m and K_m values are the mean ± S.E. of 3 experiments.

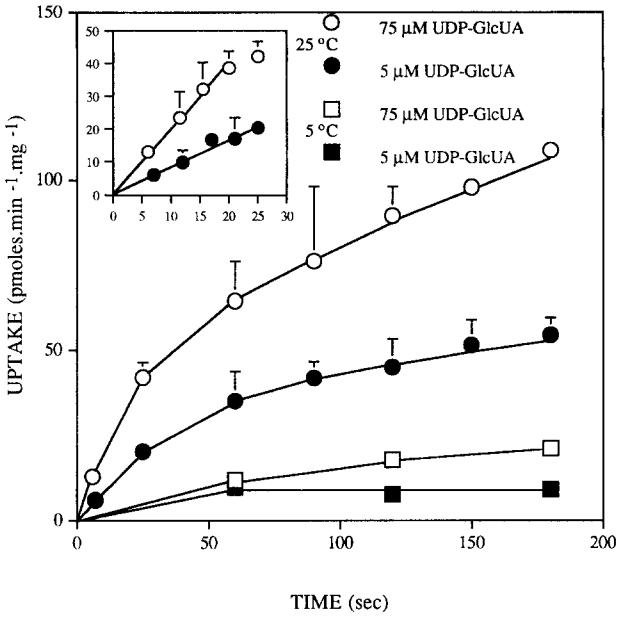

Fig. 2. Time and temperature-dependence of [β-^{32}P]UDP-GlcUA transport into ER vesicles. Initial rates of 5 μM and 75 μM UDP-GlcUA uptake were measured at 4°C and 25°C in intact rat liver microsomal vesicles (250 μg protein), as described under Section 2. (Inset) Initial time course of uptake of 5 μM and 75 μM [β-^{32}P]UDP-GlcUA at 25°C. Values represent the mean ± S.E. of triplicate determinations from two different microsomal preparations.

3.2. Time- and temperature-dependence of UDP-GlcUA uptake

Fig. 2 shows the time-course and temperature-dependence of UDP-GlcUA transport into ER vesicles. The transport of 5 μM and 75 μM UDP-GlcUA into microsomal vesicles was linear over 25 and 20 s incubations, respectively, at 25°C (Fig. 2, inset). Uptake measurements at time periods up to 60 min reached equilibrium at 20 min and 40 min for the high and low affinity components, respectively. In both cases, uptake is followed by the initial 'overshoot' phenomenon due to a *trans*-stimulation effect by intravesicular UDP-sugars [11] (data not shown).

As shown in Fig. 2, the transport of UDP-GlcUA was strongly temperature-dependent. The uptake rates at 1 min for the low and high affinity components were decreased by 70 and 80%, respectively, when the temperature was reduced from 25°C to 4°C. The uptake of [^{14}C]UDP-GlcUA at the same concentrations and for the same time-period as those described in Fig. 2, was found to be identical to that of [β-^{32}P]UDP-GlcUA (results not shown). This observation supports the previous finding that UDP-GlcUA, and

not the product of its enzymatic hydrolysis, is the substrate being transported [11].

3.3. Evidence that osmolarity affects both components of UDP-GlcUA uptake into ER vesicles

In order to distinguish between time- and temperature-dependent binding to the ER membranes and translocation of UDP-GlcUA, the influence of osmolarity on both components of the uptake was investigated. Uptake was measured at equilibrium (40 min incubation at 37°C) in the presence of either 5 or 75 μM UDP-GlcUA under differing osmotic conditions (0.3–0.5 osm · kg^{-1}). [^{14}C]UDP-GlcUA was used instead of [β-^{32}P]UDP-GlcUA for this study because at 37°C UDP-GlcUA is extensively metabolized at the lumenal face of the ER. Bossuyt and Blanckaert postulated that the resulting UMP, as well as P_i, is transported out of the ER [13], resulting in loss of the ^{32}P label in the UDP moiety. It was also shown that, of the products of hydrolysis, the ^{14}C-labeled glucuronic acid was not transported out of the ER under these conditions. Thus, it is necessary to use [^{14}C]UDP-GlcUA when the uptake is evaluated at 37°C and equilibrium (40 min incubation). As shown in Fig. 3, the uptake of the ^{14}C-radiolabel into the ER vesicles was dependent on the osmolarity of the incubation medium for both transporting components. Moreover, plotting the uptake at the equilibrium as a function of 1/osmolarity resulted in a linear relationship. The intercept on the *y*-axis allowed the determination of a binding component (at infinite raffinose concentration) which represents 22.5% of the total incorporation at equilibrium. This is in accordance with previous work [11,12] and is independent of the UDP-GlcUA concentration used. Assuming an intravesicular volume of approximately 2 μl/mg protein, the internal UDP-GlcUA concentration at equilibrium would reach about 50 μM at an external UDP-GlcUA concentration of 5 μM and 425 μM at an external UDP-GlcUA concentration of 75 μM [11], (Berg, C. and Gollan, J., unpublished observations). These values represent concentration gradients of 10- and 5.7-fold across the microsomal membrane for the high and low affinity components, respectively. This steady-state accumulation of UDP-GlcUA further supports carrier-mediated transport into ER vesicles. The possible driving force generating the UDP-GlcUA gradient could be provided by a mechanism

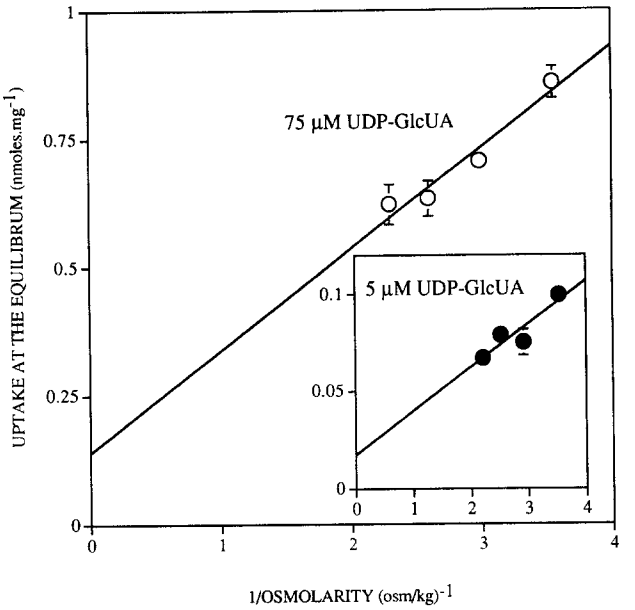

Fig. 3. Effect of osmolarity on UDP-GlcUA uptake into ER vesicles. Rat liver microsomes (250 μg protein) were incubated for 15 min at 37°C in 0.25 M sucrose, 1 mM $MgCl_2$, 10 mM Tris-HCl pH 7.4 and increasing raffinose concentrations (0–150 mM). [^{14}C]UDP-GlcUA was then added at a final concentration of 5 μM (●) or 75 μM (○) and uptake was measured, as described under Section 2. Values represent the mean ± S.E. of triplicate determinations from two different microsomal preparations.

of coupling between internal UDP-GlcNAc efflux and UDP-GlcUA influx [13].

3.4. UDP-sugars and the specificity of UDP-GlcUA uptake into ER vesicles

The specificity of the UDP-GlcUA uptake for both components was characterized by examining *cis*-inhibition and *trans*-stimulation with UDP-GlcUA, UDP-GlcNAc, UDP-Glc and UDP-Xyl. The effect of UDP-sugars in contact with the *cis*-face of the UDP-GlcUA transporter(s) (cytosolic side of the ER vesicles) was examined by externally adding the corresponding UDP-sugar at 0.1 mM and 1.0 mM concentrations, measuring the initial rate of UDP-GlcUA influx at 15 s and 25°C, and comparing it to the null *cis*-condition (uptake control with no competitor). Both components of UDP-GlcUA uptake were found to be inhibited in a dose-dependent manner by UDP-sugars (Table 1). Qualitatively, the same pattern of inhibition was

Table 1
cis-Inhibition by UDP-sugars of [β-^{32}P]UDP-GlcUA uptake into intact rat liver microsomes

	UDP-GlcUA (mM)		UDP-GlcNAc (mM)		UDP-Glc (mM)		UDP-Xyl (mM)	
	0.1	1	0.1	1	0.1	1	0.1	1
High affinity system (5 μM UDP-GclUA)	12 ± 1	5 ± 1	34 ± 6	9 ± 2	76 ± 21	42 ± 3	62 ± 17	15 ± 3
Low affinity system (75 μM UDP-GlcUA)	43 ± 5	29 ± 2	82 ± 0	35 ± 7	93 ± 24	77 ± 21	78 ± 7	63 ± 2

The [β-^{32}P]UDP-GlcUA uptake into ER vesicles (250 μg proteins) was measured at 25°C after 15-s incubation in the presence of externally added UDP-GlcUA, UDP-GlcNAc, UDP-Glc, UDP-Xyl at 0.1 mM and 1 mM concentrations as described under Section 2. Uptake in the absence of UDP-sugars was assigned as 100% (uptake control, zero *cis* conditions). Values represent the mean ± S.E. of triplicate determinations from two different microsomal preparations. Results are expressed as % control.

Table 2
trans-Stimulation by UDP-sugars of [β-^{32}P]UDP-GlcUA influx into intact rat liver microsomes

	UDP-GlcUA		UDP-GlcNAc		UDP-Glc		UDP-Xyl	
	0.1	1	0.1	1	0.1	1	0.1	1
High affinity system (5 μM UDP-GlcUA)	86 ± 13	113 ± 14	194 ± 59	539 ± 6 [a]	115 ± 2	132 ± 1 [a]	95 ± 10	111 ± 4
Low affinity system (75 μM UDP-GlcUA)	112 ± 7	134 ± 28	100 ± 26	232 ± 49	94 ± 11	111 ± 9	98 ± 12	114 ± 22

ER microsomal vesicles were preloaded with UDP-GlcUA, UDP-GlcNAc, UDP-Glc, UDP-Xyl at 0.1 mM and 1 mM concentrations throughout the microsomal preparations. The initial uptake of [β-^{32}P]UDP-GlcUA (50 μg proteins) in the absence of preloaded UDP-sugars was assigned as 100% (control). The percentage of residual uptake (*trans*-stimulation) was expressed after correction for the *cis*-inhibition due to extravesicular UDP-sugars (1.4–14 μM) in the incubation medium. Values represent the mean ± S.E. of triplicate determinations from two different microsomal preparations. Results are expressed as % control.
[a] significantly different from control (zero *trans* condition) with $P < 0.05$.

observed for both transporting components, while quantitatively, the high affinity component showed a stronger inhibition by UDP-sugars as compared to the low affinity system under the experimental conditions employed. After UDP-GlcUA, UDP-GlcNAc was the most potent inhibitor, followed by UDP-Xyl and UDP-Glc, suggesting that these compounds might be substrates (with varying affinities) for the transporter. It has been previously shown that UDP-GlcUA is a *cis*-inhibitor of UDP-Glc influx and a *trans*-stimulator of the UDP-Glc efflux [23], and these two UDP-sugars might thus share, at least in part, the same transporter. The *cis*-addition of non-labeled UDP-GlcUA led to a sharp decrease of vesicle-associated radioactivity, in accordance with a carrier-mediated process for both uptake components (Table 1). The *trans*-stimulation of UDP-GlcUA uptake into ER vesicles by these UDP-sugars was investigated by preloading the vesicles (0.1 and 1.0 mM sugar-nucleotide) and measuring the UDP-GlcUA influx relative to the proper uptake control (non-preloaded microsomes to which were added UDP-sugars at the concentrations determined to be present at the *cis*-face of the pre-loaded microsomes during influx measurements). Of the nucleotide sugars tested, UDP-GlcNAc was found to be the most efficient in *trans*-stimulating UDP-GlcUA import: influx of 5 μM and 75 μM UDP-GlcUA were stimulated up to 5 times and 2 times, respectively, when microsomal vesicles were preloaded with 1.0 mM UDP-GlcNAc (Table 2). UDP-Glc and UDP-Xyl did not show any significant *trans*-stimulatory effect. *trans*-Stimulation experiments with UMP showed no effect on either component of UDP-GlcUA uptake (Table 3).

Table 3
Effect of preloading intact rat liver microsomes with UMP on the [β-^{32}P]UDP-GlcUA influx

	UMP (mM)	
	0.1	1
High affinity system	96 ± 19	120 ± 15
Low affinity system (75 μM UDP-GlcUA)	92 ± 13	82 ± 7

ER microsomal vesicles were preloaded with UMP at 0.1 mM and 1 mM concentrations throughout the microsomal preparations. The initial uptake of [β-^{32}P]UDP-GlcUA (50 μg protein) in the absence of preloaded UMP was assigned as 100% (control). The percentage of residual uptake was expressed after correction for the *cis*-inhibition due to extravesicular UMP (1.4–14 μM) in the incubation medium. Values represent the mean ± S.E. of triplicate determinations from two different microsomal preparations. Results are expressed as % control.

3.5. Effect of anion-transport inhibitors on UDP-GlcUA uptake into ER vesicles

Different classes of inhibitors were used to characterize both components of the UDP-GlcUA uptake. It has been shown previously that SITS and DIDS inhibit the carrier-mediated transport of UDP-GlcUA, i.e., what we now identify as the high affinity component [10,12]. In the present studies, we investigated the effect of SITS and DIDS on both uptake components in intact microsomes. The evaluation of IC_{50} values for both compounds presented in Table 4 clearly indicates that DIDS does not discriminate, while the inhibition by SITS was approximately three times more pronounced for the high affinity component of the uptake as compared to the low affinity component.

Diuretics, such as probenecid and furosemide, are used widely as reversible inhibitors of anion carriers [24].

Table 4
Effect of SITS and DIDS on the uptake of [β-^{32}P]UDP-GlcUA into intact microsomes

	IC_{50} DIDS (μM)	IC_{50} SITS (μM)
High affinity system (5 μM UDP-GlcUA)	149 ± 19	363 ± 20 [a]
Low affinity system (75 μM UDP-GlcUA)	162 ± 45	1106 ± 9 [a]

Microsomal vesicles (21.3 mg proteins/ml) were incubated for 10 min with different concentrations of SITS (0.05–1.2 mM), DIDS (0.05–0.8 mM) or 2% (v/v) DMSO (control) at 25°C. Microsomal vesicles were diluted 10 times in 0.25 M sucrose, 1 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4 and initial rates of UDP-GlcUA uptake in intact rat liver microsomes were measured at 5 μM ('high affinity system') and 75 μM ('low affinity system') radiolabeled UDP-GlcUA after 15-s incubation at 25°C as described under Section 2. IC_{50} is the concentration of the irreversible inhibitors SITS and DIDS which causes 50% inhibition of the uptake of 5 and 75 μM radiolabeled UDP-GlcUA. Values represent the mean ± S.E. of triplicate determinations from two different microsomal preparations.
[a] Significantly different between high and low affinity components ($P < 0.05$).

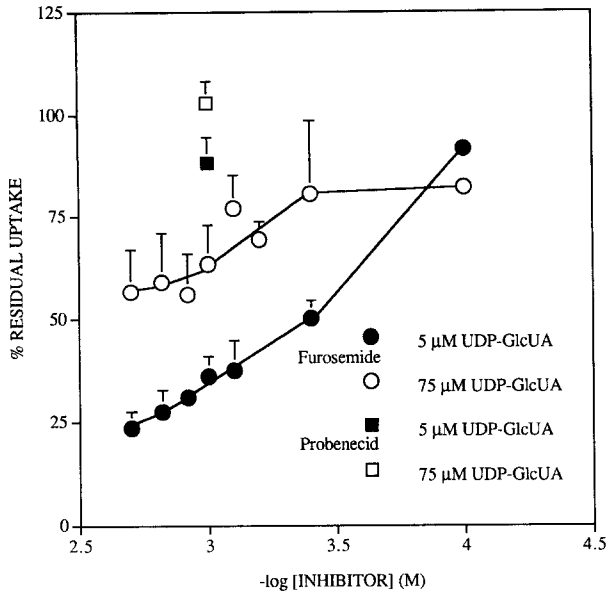

Fig. 4. Inhibition of the uptake of [β-^{32}P]UDP-GlcUA by furosemide and probenecid. Initial rates of UDP-GlcUA uptake in intact rat liver microsomes (250 μg protein) were measured at 5 μM and 75 μM radiolabeled UDP-GlcUA after 15-s incubation at 25°C with different concentrations of furosemide (0.1–2 mM) or with 1 mM probenecid, as described under Section 2. The percentage of residual uptake is the ratio of the uptake in the presence of inhibitors compared to a control uptake (100%) in which inhibitors were omitted. Values represent the mean ± S.E. of triplicate determinations from two different microsomal preparations.

Therefore, the inhibition pattern of the UDP-GlcUA uptake by these inhibitors was determined. Fig. 4 shows the inhibition for the two UDP-GlcUA uptake components by these reversible anion-transport inhibitors. The inhibition by furosemide was tested at a concentration range of 0.1–2.0 mM. The inhibitory effect of furosemide is diminished when the uptake is performed at 75 μM UDP-GlcUA compared to 5 μM, suggesting that the low affinity component is less sensitive to these inhibitors than the high affinity component. The inhibitory effect of probenicid was examined only at a concentration of 1 mM due to its limited solubility and no inhibition was observed.

Table 5
Effect of AZT and AZTMP on the initial uptake rate of 5 μM and 75 μM [β-^{32}P]UDP-GlcUA into intact microsomes

	AZT (mM)		AZTMP (mM)	
	0.1	1	0.1	1
High affinity system (5 μM UDP-GlcUA)	85 ± 7	79 ± 14	60 ± 7	40 ± 10
Low affinity system (75 μM UDP-GlcUA)	98 ± 3	96 ± 1	90 ± 3	57 ± 3

The [β-^{32}P]UDP-GlcUA uptake into ER vesicles (250 μg proteins) was measured at 25°C after 15-s incubation as described under Section 2. The uptake in the absence of inhibitors was assigned as 100% (control). Values represent the mean ± S.E. of triplicate determinations from two different microsomal preparations. Results are expressed as % control.

3.6. AZTMP inhibits UDP-GlcUA transport into the ER vesicles

A recent study has shown that AZTMP, the primary metabolite of the chemotherapeutic agent AZT, inhibits protein glycosylation in Golgi membranes of CHO cells by competitive binding to several nucleotide-sugar carriers [25]. For this reason, we evaluated the possible inhibitory effect of AZT and AZTMP (0.1 mM and 1.0 mM) on both the high and low afffinity components of UDP-GlcUA uptake in microsomal vesicles. As shown in Table 5, AZT did not inhibit the uptake of either concentration of UDP-GlcUA. However, AZTMP exhibited a dose-dependent inhibition of both components of UDP-GlcUA uptake, with 50% inhibition in the range of 1 mM.

4. Discussion

Early investigations suggested that UDP-GlcUA transport is a carrier-mediated process [9]. Studies in intact ER vesicles have subsequently provided a functional characterization of UDP-GlcUA transport [10–12].

The data presented here demonstrate that in addition to a transport system characterized by a low K_m, UDP-GlcUA is a substrate for at least one other transport component. UDP-GlcUA uptake by intact ER vesicles was mediated by both a high affinity (K_m 1.6 μM) and a low affinity (K_m 38.0 μM) transport system, but only the high affinity component has been detected in previous studies [10,12]. Because the prior experiments were performed at UDP-GlcUA concentrations of 3.5–5.0 μM, the contribution of the low affinity component to the transport of UDP-GlcUA was negligible, and the characteristics of the system described correspond to the high affinity transport component in the present studies. However, under the present experimental conditions, the V_m of the high affinity transporting component was lower than previously described [12].

Recently, Bossuyt et al., performed extensive characterization of the UDP-GlcUA uptake process in rat liver ER [11]. In most experiments, the average concentration of UDP-GlcUA used for the characterization of UDP-GlcUA uptake was 25 μM; hence, this characterization of UDP-GlcUA uptake is likely to represent the overlapping contributions of both high and low affinity components. The authors were unable to provide conclusive evidence for two components of the transport of UDP-GlcUA, based on the kinetic parameters observed in those studies [11]. However, several multi-component models for carrier-mediated transport of various ligands across membranes, including the ER, have been described previously [23,26,27]. Our studies indicate that the two-component system for the transport of UDP-GlcUA may belong to this class of transporters.

In order to present definitive proof that a new UDP-GlcUA transport component had been detected, it was necessary to document that the transport system was an integral part of the ER, rather than originating from contamination by other subcellular fractions. The unavoidable presence of the Golgi marker enzyme, ovomucoid UDP-galactosyltransferase, has been reported previously in ER preparations used for UDP-GlcUA transport studies [11,21]. The activity of this enzyme in our microsomal preparation was 4.87 ± 0.19 nmol/h · mg protein and was in the range of previously published data [21]. However, we have accumulated evidence which excludes the possibility that either of the UDP-GlcUA components originate from Golgi contamination. The functional characterization of UDP-GlcUA transport in Golgi vesicles and Golgi protein proteoliposomes has been well-characterized [28]. The apparent K_m of the transport was in the range of 3–5 μM and the uptake was significantly stimulated by UMP. However, it has been shown previously that the high affinity component in ER vesicles, which is characterized by a K_m similar to that observed in the Golgi vesicles, is not *trans*-stimulated by UMP [12] and this observation was confirmed in the course of the present study (Table 3). The low affinity component is characterized by an apparent K_m value approximately ten times higher than that in Golgi membranes and is not *trans*-stimulated by UMP (Table 3). The Golgi transporting system is characterized by a low uptake rate [28], in contrast to the relatively high velocities of both components identified and characterized in our studies (Fig. 1).

The major focus of the present studies was a detailed comparison of the high and low affinity transporting components. Substrate specificity determination, time- and temperature-dependence, various inhibitory studies and osmolarity dependence were performed simultaneously for both systems. Substrate specificity studies of the two components indicated that other UDP-sugars are *cis*-inhibitors of UDP-GlcUA uptake (Table 1). Thus, the same transporter may contribute to the translocation of other sugar nucleotides, possibly as an alternate transport system regulating their availability for conjugation reactions within the lumen of the ER.

For more detailed comparative studies, various inhibitors of anion transport were employed to further differentiate the two uptake components. Stilbene disulfonate derivatives, such as SITS and DIDS, covalently bind through their isothiocyano groups to the ε-amino group of lysyl residues of membrane carriers. SITS and DIDS have a very similar structure, but DIDS possesses two reactive isothiocyanate groups, while SITS has only one and, thus, DIDS can potentially provide a cross-linkage between two lysyl residues. In our studies, where the IC_{50} for both inhibitors of both components was evaluated, this property strengthens the inhibitory potency of DIDS compared to SITS (Table 4). SITS and DIDS are membrane-impermeant compounds and their binding site(s) is (are) thus likely to be on the cytosolic face of the UDP-GlcUA transporter(s). Since a higher UDP-GlcUA concentration increases the SITS IC_{50} value, it is reasonable to assume that SITS competes with UDP-GlcUA for the same binding site if a large fraction of its inhibitory effect is reversible. Such a partial reversibility of SITS binding has been observed for other transport systems [29].

Two reversible inhibitors of anion transporters, probenicid and furosemide, were tested for their ability to inhibit both transport components (Fig. 4). Probenicid did not exhibit any significant inhibition of either component, whereas furosemide showed a dose-dependent inhibition of uptake by both components. The high affinity component was potently inhibited, while the low affinity compound was less sensitive to this inhibitor in the same concentration range, which supports a partial or total overlapping of the binding sites for furosemide and UDP-GlcUA. Finally, the effect of inhibition of UDP-GlcUA transport by AZT and its primary metabolite, AZTMP, were examined. It was shown recently that AZTMP is a potent competitive inhibitor of the influx of nucleotide sugars into the Golgi apparatus [25]. Our studies, as shown in Table 5, demonstrated that AZTMP, but not AZT, inhibited UDP-GlcUA influx into ER vesicles. Furman et al. [30] demonstrated that the phosphorylation of AZT is catalyzed by a cytosolic thymidine kinase, and AZTMP was found to be at a concentration close to 1 mM in a human cell line. Thus, it is reasonable to assume that in vivo AZTMP could inhibit UDP-GlcUA transport and subsequent glucuronidation reactions by a partial depletion of UDP-GlcUA within the lumen of the ER. Moreover, AZT also inhibits the formation of uridine nucleotides [31] involved in early stages of UDP-GlcUA synthesis. It has also been demonstrated that AZT is extensively glucuronidated in human liver microsomes and competes with the glucuronidation of other drugs and steroid hormones [32]. Recently, we have utilized a photoaffinity analog of AZTMP in rat liver microsomes to identify a 130 kDa protein that interacts with AZTMP and UDP-sugars [33]. Therefore, multiple effects of AZT and its metabolite AZTMP on UGTs and UDP-GlcUA transport could impair normal glucuronidation of endogenous and exogenous compounds.

A long-standing question in hepatic glucuronidation is the involvement of UDP-GlcNAc as a physiological activator. In our studies, both components of the UDP-GlcUA uptake were *trans*-stimulated and *cis*-inhibited by UDP-GlcNAc (Tables 1 and 2). The significant *trans*-stimulation of both components provided strong evidence that the translocation of UDP-GlcUA across the ER is a facilitated diffusion process. More interestingly, UDP-GlcUA itself was not efficient in *trans*-stimulating the uptake compared to UDP-GlcNAc. Two models have been proposed for the carrier-mediated uptake of UDP-GlcUA into the ER. The *first model* includes a transporter with a shared specificity towards UDP-GlcUA and UDP-GlcNAc, and is based on the potent stimulatory effect of an outwardly-directed

UDP-GlcNAc gradient on the influx of UDP-GlcUA, which suggests that the transporter might have an high affinity for UDP-GlcNAc. The recently proposed *second model* includes an antiporter which couples the UDP-GlcUA entering the ER with the efflux of UDP-GlcNAc [13]. Our data supports this second model with UDP-GlcUA, UDP-Glc, UDP-Xyl as *cis*-inhibitors but not *trans*-stimulators of UDP-GlcUA influx. This model also is supported by our studies where a potent *cis*-inhibitory/*trans*-stimulatory effect of UDP-GlcNAc on UDP-GlcUA import (Tables 1 and 2) was observed. However, it is premature to propose reliable models based on these available data. The identification and isolation of the protein(s) involved and reconstitution followed by direct transport measurements with both UDP-GlcUA and UDP-GlcNAc would answer this question definitively.

In summary, we have provided evidence for the existence of two UDP-GlcUA transport systems in rat liver ER. In general, these two components for the transport of UDP-GlcUA share major properties. The similarity of the patterns of inhibition of the two systems by anion-transport inhibitors, as well as *cis*-inhibition and *trans*-stimulation by UDP-sugars, suggest structurally and functionally related transporters. As previously emphasized for other transport systems [26], it is unclear whether these two components reflect the presence of one protein with two affinities for UDP-GlcUA or multiple transporter proteins. Studies are in progress to identify the proteins involved.

Acknowledgements

This work was supported in part by an NIH grants DK-38678 (to A.R.), DK-45123 (to R.L.), DK-36887 (to J.G.) and DK-02168 (to C.B.). We wish to thank Dr P. Zimniak and J.M. Little for their critical evaluation of the manuscript.

References

[1] Dutton, G.J. (1980) Glucuronidation of Drugs and Other Compounds, CRC Press, Boca Raton, FL.
[2] Burchell, B., Nebert, D.W., Nelson, D.R., Bock, K.W., Iyanagi, T., Jansen, P.L.M., Lancet, D., Mulder, G.J., Roy Chowdhury, J., Siest, G., Tephly, T.R. and Mackenzie, P.I. (1991) DNA Cell Biol. 10, 487–494.
[3] Vanstapel, F. and Blanckaert, N. (1988) Arch. Biochem. Biophys. 263, 216–225.
[4] Shepherd, S.R.P., Baird, S.J., Hallinan, T. and Burchell, B. (1989) Biochem. J. 259, 617–620.
[5] Yokota, H., Yuasa, A. and Sato, R. (1992) J. Biol. Chem. 112, 192–196.
[6] Drake, R., Igari, I., Lester, R., Elbein, A. and Radominska, A. (1992) J. Biol. Chem. 267, 11360–11365.
[7] Fulceri, R., Banhegyi, G., Gamberucci, A., Giunti, R., Mandl, J. and Benedetti, A. (1994) Arch. Biochem. Biophys. 309, 43–46.
[8] Winsnes, A. (1972) Biochim. Biophys. Acta 284, 394–405.
[9] Berry, C. and Hallinan, T. (1976) Biochem. Soc. Trans. 4, 650–652.
[10] Hauser, S.C., Ziurys, J.C. and Gollan, J.L. (1988) Biochim. Biophys. Acta 967, 149–157.
[11] Bossuyt, X. and Blanckaert, N. (1994) Biochem. J. 302, 261–269.
[12] Berg, C., Radominska, A., Lester, R. and Gollan, J. (1995) Gastroenterology 108.
[13] Bossuyt, X. and Blanckaert, N. (1995) Biochem. J. 305, 321–328.
[14] Radominska, A., Berg, C., Treat, S., Little, J.M., Gollan, J., Lester, R. and Drake, R. (1994) Biochim. Biophys. Acta 1195, 63–70.
[15] Radominska, A. and Drake, R. (1994) Methods Enzymol. 230, 330–339.
[16] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[17] Arion, W.J. (1989) Methods Enzymol. 174, 58–67.
[18] Blair, J.N.R. and Burchell, A. (1988) Biochim. Biophys. Acta 964, 161–167.
[19] Scharschmidt, B.F., Keeffe, E.B., Blankenship, N.M. and Ockner, R.K. (1979) J. Lab. Clin. Med. 93, 790–799.
[20] Bretz, R. and Staubli, W. (1977) Eur. J. Biochem. 77, 181–192.
[21] Hauser, S.C., Ziurys, J.C. and Gollan, J.L. (1984) J. Biol. Chem. 259, 4527–4533.
[22] Bossuyt, X. and Blanckaert, N. (1994) Eur. J. Biochem. 223, 981–988.
[23] Vanstapel, F. and Blanckaert, N. (1988) J. Clin. Invest. 82, 1113–1122.
[24] Aronson, P.S. (1989) Annu. Rev. Physiol. 51, 419–41.
[25] Hall, E.T., Yan, J.-P., Melancon, P. and Kuchta, R.D. (1994) J. Biol. Chem. 269, 14355–14358.
[26] Ballatori, N. and Dutczak, W.J. (1994) J. Biol. Chem. 269, 19731–19737.
[27] Dutczak, W.J. and Ballatori, N. (1994) J. Biol. Chem. 269, 9746–9751.
[28] Milla, M.E., Clairmont, C.A. and Hirschberg, C.B. (1992) J. Biol. Chem. 267, 103–107.
[29] McConnell, K.R. and Aronson, P.S. (1994) J. Biol. Chem. 269, 21489–21494.
[30] Furman, P.A., Fyfe, J.A., St Clair, M.H., Weinhold, K., Rideout, J.L., Freeman, G.A., Nusinoff Lehrman, S., Bolognesi, D.P., Broder, S., Mitsuya, H. and Barry, D.W. (1986) Proc. Natl. Acad. Sci. USA 83, 8333–8337.
[31] Faraj, A., Naguib, F. and Sommadossi, J.P. (1993) Proc. Am. Assoc. Cancer Res. 34, 354.
[32] Herber, R., Magdalou, J., Haumont, M., Bidault, R., Van Es, H. and Siest, G. (1992) Biochim. Biophys. Acta 20–24.
[33] Mao, F., Rechtin, T.M., Jones, R., Cantu, A.A., Anderson, L.S., Radominska, A., Moyer, M.P. and Drake, R.R. (1995) J. Biol. Chem. 270, 13660–13664.