MOAT4, a novel multispecific organic-anion transporter for glucuronides and mercapturates in mouse L1210 cells and human erythrocytes

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Glucuronides and mercapturates were examined as possible high-affinity substrates for a low-affinity ATP-dependent transport system for 2,4-dinitrophenyl *S*-glutathione (DNP-SG) in mouse L1210 cells. Initial inhibitor studies with inside-out vesicles revealed that the low-affinity transport of [³H]DNP-SG (K_m 450 μ M) exhibits a high sensitivity to *N*-acetyl 2,4-dinitrophenyl cysteine (NAc-DNP-Cys) (K_i 5.0 μ M) and α -naphthyl β -D-glucuronide (naphthyl glucuronide) (K_i 8.5 μ M). Direct transport measurements showed the presence of ATP-dependent uptake activities for NAc-DNP-[³⁵S]Cys and naphthyl [¹⁴C]glucuronide, and K_m values for half-maximal transport were comparable to the K_i values of these compounds for inhibition of [³H]DNP-SG transport. Transport of [³H]DNP-SG, NAc-DNP-[³⁵S]Cys and naphthyl [¹⁴C]glucuronide each showed the same sensitivity to various anions and anion conjugates. Inhibition was

competitive and was most potent for bilirubin ditaurate, indoprofen, 4-biphenylacetic acid, 4-acridine 4 β -D-glucuronide, *N*-acetyl leukotriene E₄, 17 β -oestradiol 3 β -D-glucuronide and taurolithocholate 3-sulphate. Inside-out vesicles from human erythrocytes contain a comparable ATP-dependent transport system. These results show that NAc-DNP-Cys and naphthyl glucuronide are high-affinity substrates for a single system identified previously as a low-affinity transporter of DNP-SG. Substrate and inhibitor studies identify this system as a novel multispecific organic-anion transport system (MOAT4) that accommodates glucuronides and mercapturates and is distinct from other MOAT transporters. Human erythrocytes contain an additional ATP-dependent system for NAc-DNP-Cys (K_m 33 μ M) that does not transport monoglucuronides.

INTRODUCTION

Xenobiotics and antitumour drugs can be converted by intracellular metabolism to detoxified hydrophilic conjugates, which are eliminated by ATP-dependent efflux pumps. Efflux systems are also employed to expel various metabolically derived anions and anionic conjugates that are involved in physiological functions as diverse as vasodilation [1], extracellular signalling [2–4], and ion channel regulation [5,6]. Conjugates formed by cells often arise from coupling with glutathione or glucuronic acid at one or multiple sites, and glutathione conjugates (GS-conjugates) can be metabolized further to various mercapturates (*N*-acetylcysteine conjugates) [7,8]. Efflux activities for GS-conjugates [2,9–11], glucuronides [11–15] and mercapturates [12,16] have been described in various cells and tissues, and in some cases multiple kinetic routes have been identified [17–21]. A general feature of these efflux activities is a broad specificity, as determined from substrate and inhibitor studies [11,18,20] and from a loss in transport activity for various conjugates in canalicular membrane vesicles from TR[−] rats with hyperbilirubinaemia [11,22–25]. This broad specificity has led to the designation of ATP-dependent efflux systems for anionic conjugates as multi-specific organic-anion transporters (MOATs) [11,26,27].

Recent studies have established that various cells and tissues contain genetically distinct ATP-dependent efflux systems for anions and anionic conjugates. Liver canalicular MOAT (cMOAT) is the primary GS-conjugate pump in normal liver canalicular membranes, and is defective in TR[−] rats [11,23].

From deficiencies in TR[−] rats, this system was found to have an unusually broad specificity and to mediate the extrusion of conjugates with glutathione, glucuronic acid and other compounds [11]. cMOAT has been sequenced from isolated cDNA [28], contains a single nucleotide deletion in TR[−] rats [28] and is structurally similar to, but distinct from, multidrug resistance-associated protein (MRP) [29]. The transport specificities of cMOAT and MRP are similar [30]. Studies have also shown that the GS-conjugate pump in erythrocytes from TR[−] rats is normal [24], indicating that haematopoietic cells and liver contain genetically distinct efflux systems for GS-conjugates.

L1210 mouse leukaemia cells express two ATP-dependent efflux activities for 2,4-dinitrophenyl *S*-glutathione (DNP-SG) that have distinct differences in substrate affinity and can be measured separately with specific inhibitors [20]. The high-affinity system for DNP-SG (K_m 0.63 μ M) exhibits a tight binding for various GS-conjugates and bilirubin ditaurate, and has the added flexibility to transport cholate [20] and methotrexate [31]. The low-affinity system for DNP-SG (K_m 450 μ M), which also transports methotrexate [31], binds various GS-conjugates poorly or not at all [20] but has a high affinity for monovalent and divalent anions such as 4-biphenylacetic acid (BPAA), indoprofen and bilirubin ditaurate [20]. The two transport systems for DNP-SG in L1210 cells seem to be genetically distinct because variant cells defective in the high-affinity system exhibit normal transport via the low-affinity system [20]. High- and low-affinity transport systems for DNP-SG have also been identified in vesicles from human erythrocytes [17,18].

The present study was initiated to identify physiological

Abbreviations used: BPAA, 4-biphenylacetic acid; CDNB, 1-chloro-2,4-dinitrobenzene; cMOAT, liver canalicular MOAT; DNP-SG, 2,4-dinitrophenyl *S*-glutathione; EA-SG, glutathione conjugate of ethacrynic acid; h, human; GS-conjugate, glutathione conjugate; m, mouse; MOAT, multispecific organic-anion transporter; MRP, multidrug resistance-associated protein; NAc-BSP-Cys, *N*-acetylcysteine conjugate of bromosulphthalein; NAc-DNP-Cys, *N*-acetyl 2,4-dinitrophenyl cysteine; NAc-DNP-SG, *N*-acetyl DNP-SG; NAc-EA-Cys, *N*-acetylcysteine conjugate of ethacrynic acid; NAc-LTE₄, *N*-acetyl leukotriene E₄; r, rat.

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substrates for the low-affinity transport system for DNP-SG in L1210 cells. Since previous transport studies had identified certain monovalent anions as potent inhibitors of this system [20], monoanionic conjugates were examined as possible substrates. The results identify glucuronides and mercapturates as primary physiological substrates for the low-affinity efflux system for DNP-SG in L1210 cells. Comparative studies show that human erythrocytes contain the same transport activity. Substrate and inhibitor specificity indicates further that this system is a MOAT that differs from MOAT transport systems described previously. Designation as MOAT4 has been adopted to distinguish this activity from other known conjugate transport activities that fit the MOAT phenotype but are kinetically and/or genetically distinct. For descriptive purposes, separate MOAT designations are employed for the activities of cMOAT (MOAT1) and MRP (MOAT2), and the high-affinity transporter for GS-conjugates in rat and human erythrocytes and L1210 leukaemia cells (MOAT3).

MATERIALS AND METHODS

Chemicals

[glycine-2-³H]Glutathione (44 Ci/mmol) and L-[³⁵S]cysteine (1075 Ci/mmol) were obtained from New England Nuclear; UDP-[¹⁴C]glucuronic acid (300 mCi/mmol) was obtained from American Radiolabeled Chemicals. Glutathione S-transferase, glutathione, ATP, creatine phosphate, creatine kinase, *N*-acetyl cysteine, bromosulphthalein, 1-chloro-2,4-dinitrobenzene (CDNB), ethacrynic acid, flurbiprofen, indoprofen, BPAA, 4-hydroxyacridine, taurocholate, tauroolithocholate, tauroolithocholate 3-sulphate, glycolithocholate 3-sulphate, phenyl β -D-glucuronide, 4-nitrophenyl β -D-glucuronide, α -naphthyl β -D-glucuronide, UDP glucuronic acid, UDP glucuronyl transferase (type II, rabbit liver), β -glucuronidase (*Escherichia coli*), 17 β -oestradiol 3 β -D-glucuronide, β -oestradiol 17 β -D-glucuronide, 17 β -oestradiol 3 β -D-glucuronide 17-sulphate, androsterone 3 β -D-glucuronide, vincristine, daunorubicin (daunomycin) and dithioerythritol were obtained from Sigma. Bilirubin ditaurate was purchased from United States Biochemicals. Immobilized wheatgerm agglutinin was prepared by coupling wheatgerm agglutinin (Boehringer-Mannheim) with CNBr-activated Sepharose 4B (Sigma) [32].

DNP-SG and the glutathione conjugate of ethacrynic acid (EA-SG) were synthesized chemically, whereas [³H]DNP-SG was synthesized enzymically by procedures described previously [20]. *N*-acetyl DNP-SG (NAC-DNP-SG) was prepared by dissolving DNP-SG (10 mg) in 1.0 ml of 10 mM sodium phosphate, adjusting the pH to 7.5 with 2 M NaOH and adding 50 μ l of acetic anhydride. After incubation for 60 min at 23 °C, the product mixture (in 200 μ l portions) was subjected to HPLC by our standard procedure on a 0.46 cm $\times$ 25 cm Rainin microorb MV C18 reversed-phase column with a gradient of solvent A (0.1 % aqueous trifluoroacetic acid) and solvent B (0.1 % trifluoroacetic acid/10 % water/90 % acetonitrile) according to the following programme: 0–5 min, 100 % A; 5–30 min, 100 % A to 100 % B (standard gradient). NAC-DNP-SG appeared as a new peak at 22 min, represented approx. 80 % of the total absorbance at 278 nm, and was retarded relative to unreacted DNP-SG (21 min). A dried powder was obtained after freeze-drying that had a spectrum and a molar absorption coefficient (9600 M⁻¹·cm⁻¹ at 340 nm) indistinguishable from that of DNP-SG.

N-acetyl 2,4-dinitrophenyl cysteine (NAC-DNP-[³⁵S]Cys) was synthesized in a reaction mixture containing 45 μ l of [³⁵S]cysteine

(0.5 mCi), 50 μ l of 10 mM cysteine-free base (500 nmol), 100 μ l of 50 mM CDNB (5000 nmol) in ethanol, and 20 μ l of 50 mM sodium bicarbonate. The mixture was combined in ice and then incubated for 15 min at 23 °C. Acetic anhydride (20 μ l) and an additional portion (20 μ l) of 50 mM sodium bicarbonate were then added, and the incubation was continued for 15 min at 23 °C. HPLC with the standard gradient was employed to separate NAC-DNP-[³⁵S]Cys (20 min) from CDNB (24 min), other absorbing products (22 and 26 min), and [³⁵S]cysteine (3 min). The peak containing NAC-DNP-[³⁵S]Cys was dried by freeze-drying and rechromatographed to eliminate trace amounts of residual impurities. The recovery of radioactivity in the labelled product was 25–30 %.

The *N*-acetylcysteine conjugate of ethacrynic acid (NAC-EA-Cys) was prepared by combining ethacrynic acid (50 μ mol) and *N*-acetylcysteine (60 μ mol) in 1.0 ml of 10 mM sodium phosphate, adjusting the pH to 7.5 with 2 M NaOH, flushing with N₂ and incubating the mixture in a capped Eppendorf tube for 3 h at 23 °C. NAC-EA-Cys (22 min) was separated from unreacted ethacrynic acid (25 min) by HPLC (in 200 μ l aliquots) with the standard gradient, and pooled peak fractions were dried by freeze-drying. The yield was 80–90 %.

The *N*-acetylcysteine conjugate of bromosulphthalein (NAC-BSP-Cys) was prepared by dissolving BSP (10 μ mol) and *N*-acetylcysteine (25 μ mol) in 1.0 ml of 0.15 M NaHCO₃ and 200 μ l of DMSO, flushing with N₂ and incubating the mixture for 24 h at 23 °C. NAC-BSP-Cys was separated from unreacted BSP and *N*-acetylcysteine by HPLC (in 200 μ l aliquots) with the following modified gradient programme: 0–5 min, 100 % A; 5–40 min, 100 % A to 30 % B; 40–50 min, 30 % B to 100 % B. NAC-BSP-Cys was eluted earlier (34 min) than unreacted BSP (40 min). The concentration of NAC-BSP-Cys (yield 30–40 %) was obtained from the molar absorption coefficient of BSP (6050 M⁻¹·cm⁻¹) at 568 nm in 0.1 M Tris/HCl, pH 8.5.

Naphthyl [¹⁴C]glucuronide was prepared (in a 1.5 ml Eppendorf tube) by dissolving 50 μ Ci of UDP [¹⁴C]glucuronic acid (dried in vacuum) in 1.0 ml of 50 mM Tris/HCl, pH 7.5, containing 10 mM MgCl₂, 10 mg of UDP glucuronyl transferase and 1.0 μ mol of α -naphthol (in 100 μ l of DMSO). After flushing with N₂, the capped sample was mixed gently on a rocker table for 48 h at 23 °C. The sample was clarified by centrifugation at 20000 *g* for 5 min and then subjected to HPLC with the standard gradient. Naphthyl [¹⁴C]glucuronide (19 min) was eluted 4 min ahead of α -naphthol (23 min), was chromatographically indistinguishable from commercial naphthyl glucuronide, and was obtained in a radiochemical yield of 80 %.

4-Acridine glucuronide was synthesized by dissolving (in a 15 ml screw-capped tube) 25 μ mol of 4-hydroxyacridine in 2 ml of DMSO and diluting with 10 ml of 20 mM Tris/HCl, pH 7.5, containing 5 mM MgCl₂, 25 μ mol of UDP glucuronic acid and 40 mg of UDP glucuronyl transferase. After flushing with N₂, the sample was mixed gently for 48 h at 23 °C on a rocker table. After centrifugation at 20000 *g* for 5 min, the clarified supernatant was subjected to HPLC with the standard gradient programme. 4-Acridine glucuronide (16 min) was eluted 2 min ahead of 4-hydroxyacridine (18 min) and represented approx. 40 % of total absorbance at 278 nm. Peak fractions were pooled and dried by freeze-drying. Treatment of 4-acridine glucuronide (50 nmol) with 200 units of β -glucuronidase (in 0.5 ml of 100 mM potassium phosphate, pH 6.5) for 2 h at 37 °C [14] and rechromatography by the standard HPLC procedure produced a compound in a yield of more than 98 % that chromatographed identically with 4-hydroxyacridine. Naphthyl glucuronide incubated under the same conditions was converted quantitatively into a product that migrated identically with α -naphthol.

Cells

L1210 and L1210/C7 mouse leukaemia cells were grown in RPMI 1640 medium containing 3% (v/v) fetal bovine serum and 100 i.u./ml penicillin and 100 µg/ml streptomycin. Cells were harvested by centrifugation at 4 °C (500 g) and washed with PBS before storage at –80 °C. Human erythrocytes were obtained from healthy unidentified donors in 100–200 ml portions and used immediately.

Preparation of inside-out vesicles

Plasma membrane vesicles from frozen L1210 and L1210/C7 cells were prepared as described previously [20] by the method of Schaub et al. [33]. Vesiculated membranes were enriched for inside-out vesicles by a slow passage through a column of wheatgerm agglutinin linked to CNBr-activated Sepharose 4B. The final preparation was suspended at a concentration of 4 mg/ml protein in storage buffer [10 mM Tris/HCl/250 mM sucrose/1 mM dithioerythritol (pH 7.4)], divided into 1.0 ml portions and stored at –80 °C. The final preparation of vesicles was 80 ± 5% inside-out, as determined by an acetylcholinesterase accessibility assay [34].

Inside-out vesicles from human erythrocytes were isolated from freshly-drawn blood by the method of Steck and Kant [34] as modified by Awasthi et al. [35]. Enrichment for inside-out vesicles and storage were performed by the same procedures as employed for L1210 vesicles. Acetylcholinesterase inaccessibility indicated that the vesicles were 78 ± 5% inside-out.

Transport determinations in inside-out vesicles

Assay mixtures were prepared at 4 °C (in 12 mm × 75 mm siliconized glass tubes) and consisted of inside-out L1210 or L1210/C7 vesicles (100–200 µg) or erythrocyte vesicles (50–100 µg), 1.0 mM ATP, 10 mM MgCl₂, ATP-regenerating system (consisting of 10 mM creatine phosphate and 12 units of creatine kinase), [³H]DNP-SG (0.5 mCi/mmol), NAc-DNP-[³⁵S]Cys (0.5–1.0 mCi/mmol) or naphthyl [¹⁴C]glucuronide (0.3 mCi/mmol), the desired additions, and assay buffer [10 mM Tris/250 mM sucrose (pH 7.4)] in a final volume of 150 µl. After incubation at 37 °C, uptake was stopped by placing on ice. The vesicles were diluted with 1 ml of ice-cold assay buffer, collected by rapid filtration on a Millipore HAWP 0.45 µm filter, washed with four 1 ml portions of ice-cold assay buffer, placed in scintillation vials containing 8 ml of scintillation fluid (Scintisafe, Fisher), and left for 16 h at 23 °C before analysis for radioactivity. Samples incubated at 37 °C without ATP served as the control. IC₅₀ values were derived from Dixon plots of the reciprocal of transport activity against inhibitor concentration. Inhibition constants (*K_i* values) were calculated by the Dixon equation:

$$-IC_{50} \text{ (x intercept)} = K_i(1 + [S]/K_m)$$

where [S] is the substrate concentration. *K_m* and *V_{max}* values were derived from Lineweaver–Burk plots of transport at varying concentrations of substrate. Two-component transport systems were separated by computer analysis with the equation:

$$v = V_{max1}/(1 + K_{m1}/[S]) + V_{max2}/(1 + K_{m2}/[S])$$

where *v* is the transport velocity. Experimental points were measured in duplicate, and kinetic values were the means of two or more separate determinations. Experiments were repeated until the standard deviation varied by less than 30%.

Protein concentrations were determined by the Bradford method [36] with BSA as the standard.

RESULTS

Effect of DNP-SG analogues and aromatic glucuronides on transport by mMOAT3 and mMOAT4 in inside-out vesicles from L1210 cells

Inside-out vesicles from mouse L1210 cells contain distinct high- and low-affinity ATP-dependent transport systems for DNP-SG [20]. The high-affinity system (designated mMOAT3; see the Discussion section) can be measured with relative ease by using radiolabelled GS-conjugates, such as [³H]DNP-SG, but assay of the low-affinity route (designated mMOAT4) has been hampered by the lack of a specific substrate. mMOAT4 had been measured with some success by using [³H]DNP-SG as the substrate in an L1210/C7 subline with a defective mMOAT3, but significant interference from mMOAT3 was observed in attempts to measure mMOAT4 activity in parental cells [20]. Because mMOAT4 exhibits a relatively high sensitivity to BPAA, indoprofen and other monovalent anions [20], the possibility was pursued that physiological substrates for this system might include mercapturates, monoglucuronides and/or bile acid conjugates.

Inhibitor studies were performed with [³H]DNP-SG as the substrate in an effort to identify anions with a high affinity for mMOAT4 and a low affinity for mMOAT3. Conversion of the zwitterionic DNP-SG into the dianionic NAc-DNP-SG resulted in a decrease in affinity for mMOAT3 to one-eighteenth, but no detectable improvement was observed in binding to mMOAT4 (Table 1). However, a profound change in specificity was observed by replacing the glutathione portion of NAc-DNP-SG with cysteine (Table 1). NAc-DNP-Cys showed little or no affinity for mMOAT3 (*K_i* 500 µM) but had a high affinity for mMOAT4 (*K_i* 5.0 µM).

Aromatic glucuronides were also examined for selective inhibition of mMOAT3 and mMOAT4. Phenyl, *p*-nitrophenyl and α-naphthyl β-D-glucuronides were each unable to inhibit

Table 1 Comparative responses to anion conjugates by the high-affinity (mMOAT3) and low-affinity (mMOAT4) transport systems for DNP-SG in inside-out vesicles from mouse L1210 cells

mMOAT3 (high-affinity transport system for DNP-SG) was measured (10 min at 37 °C) in vesicles from parental L1210 cells with 1.5 µM [³H]DNP-SG as the substrate in the presence of 40 µM BPAA to block a small contribution (10%) to total transport by mMOAT4 [20]. mMOAT4 (low-affinity transport system for DNP-SG) was measured (10 min at 37 °C) in vesicles from the L1210/C7 cell line defective in mMOAT3, increasing the concentration of [³H]DNP-SG to 5.0 µM and adding 40 µM EA-SG to block residual mMOAT3 activity [20]. *K_i* values were calculated by using *K_m* values of 0.63 and 450 µM for mMOAT3 and mMOAT4 respectively. Abbreviation: n.d., not determined.

Inhibitor	<i>K_i</i> (µM)	
	High-affinity system (mMOAT3)	Low-affinity system (mMOAT4)
DNP-SG	0.60	450
NAc-DNP-SG	11	500
NAc-DNP-Cys	> 500	5.0
Phenyl glucuronide	> 500	> 500
<i>p</i> -Nitrophenyl glucuronide	> 500	300
Naphthyl glucuronide	> 500	8.5
Taurocholate	150	n.d.
Tauroolithocholate	4.7	n.d.
Tauroolithocholate 3-SO ₄	0.59	n.d.
Glycolithocholate 3-SO ₄	0.44	n.d.

Figure 1 Time dependence at 37 °C for the ATP-dependent uptake of NAc-DNP-[³⁵S]Cys (A) and naphthyl [¹⁴C]glucuronide (B) by inside-out vesicles from mouse L1210 cells and human erythrocytes

The substrate concentration was 5.0 μ M; the ATP concentration was 1.0 mM.

mMOAT3, and phenyl and *p*-nitrophenyl β -D-glucuronide had no effect on mMOAT4; however, mMOAT4 showed a high affinity for naphthyl glucuronide (K_i 8.5 μ M) (Table 1). Oestradiol 17-glucuronide also failed to inhibit mMOAT3 (K_i 200 μ M) (results not shown) but was a substantial inhibitor of mMOAT4 (see Table 3). Bile acid conjugates were found to inhibit both transport systems. Relative affinities for mMOAT3 were: glycolithocholate 3-sulphate > tauroolithocholate 3-sulphate > tauroolithocholate > taurocholate (Table 1). These compounds inhibited mMOAT4 with the same order of effectiveness, albeit with a somewhat lower affinity (see Table 4), and hence were not likely candidates for specific substrates of mMOAT4. Daunorubicin and vincristine at concentrations up to 200 μ M were weak inhibitors of mMOAT3 activity; respective K_i values were 125 and 75 μ M.

Transport of NAc-DNP-Cys by inside-out vesicles from L1210 cells and human erythrocytes

Inhibitor studies (Table 1) suggested that NAc-DNP-Cys might be a specific substrate for mMOAT4 in L1210 cells. To examine this possibility, NAc-DNP-[³⁵S]Cys was synthesized and ATP-dependent transport was measured in inside-out vesicles. Vesicles from L1210 cells accumulated substantial amounts of NAc-DNP-[³⁵S]Cys by a process that was linear for 2 min at 37 °C and then reached a steady state after 15 min (Figure 1A). Omission of ATP blocked the ability of the vesicles to accumulate NAc-DNP-[³⁵S]Cys. In human erythrocyte vesicles a similar profile was obtained for the time-dependent uptake of NAc-DNP-[³⁵S]Cys, except that the extent of uptake increased by 1.8-fold (Figure 1A).

Transport of naphthyl glucuronide by inside-out vesicles from L1210 cells and human erythrocytes

Specific inhibition of mMOAT4 by naphthyl glucuronide (Table 1) led to the synthesis of naphthyl [¹⁴C]glucuronide and the analysis for ATP-dependent transport. Naphthyl [¹⁴C]glucuronide was readily transported into L1210 vesicles by a process that required ATP and was linear for nearly 10 min at 37 °C

Figure 2 Concentration dependence for the inhibition of NAc-DNP-[³⁵S]Cys transport by naphthyl glucuronide (A) and the inhibition of naphthyl [¹⁴C]glucuronide transport by NAc-DNP-Cys (B) in inside-out vesicles from L1210 cells

The line drawn in (A) is the inhibition predicted from the Dixon equation for a two-component system with a distribution of 96% and 4% and an IC_{50} for naphthyl glucuronide of 17 μ M for the major component and no inhibition by naphthyl glucuronide of the minor component; the line drawn in (B) is the inhibition predicted from the Dixon equation for a one-component system with an IC_{50} of 10 μ M for NAc-DNP-Cys. The substrate concentration was 5.0 μ M. The assay intervals were: NAc-DNP-[³⁵S]Cys, 2 min; naphthyl [¹⁴C]glucuronide, 5 min. The control transport rates were: NAc-DNP-[³⁵S]Cys, 40 pmol/min per mg of protein; naphthyl [¹⁴C]glucuronide, 33 pmol/min per mg of protein. The ATP concentration was 1.0 mM.

(Figure 1B). In human erythrocyte vesicles (Figure 1B), initial uptake was 3-fold higher than in L1210 vesicles, but linearity decreased after about 5 min.

Analysis for multiple transport routes for NAc-DNP-Cys and naphthyl glucuronide using transport inhibitors

The kinetics of inhibition by EA-SG, naphthyl glucuronide, NAc-DNP-Cys and indoprofen were employed to evaluate whether the transport of NAc-DNP-[³⁵S]Cys and naphthyl [¹⁴C]glucuronide proceeded via multiple routes. When measurements were performed in the presence of EA-SG (a MOAT3 inhibitor) at concentrations up to 40 μ M, no effect was seen on the ATP-dependent transport of either NAc-DNP-[³⁵S]Cys or naphthyl [¹⁴C]glucuronide in either L1210 or erythrocyte vesicles. These results eliminated a significant contribution to total transport by MOAT3.

NAc-DNP-[³⁵S]Cys transport in L1210 vesicles was inhibited progressively by increasing levels of naphthyl glucuronide, but inhibition was not complete at high concentrations of the inhibitor (Figure 2A). A Dixon plot analysis of the data fitted a two-component system with relative contributions at 5.0 μ M NAc-DNP-[³⁵S]Cys of 96% and 4% respectively. Inhibition of the predominant component was half-maximal at 17 μ M naphthyl glucuronide. In contrast, inhibition by indoprofen showed only a single component with an IC_{50} of 4.0 μ M.

In a reciprocal experiment in which the substrate and inhibitor was reversed, NAc-DNP-Cys was shown to inhibit the transport of naphthyl [¹⁴C]glucuronide (Figure 2B), but in this case inhibition was complete at high concentrations of NAc-DNP-Cys. Dixon plot analysis showed that inhibition fitted the predicted response of a single component with an IC_{50} of 10 μ M. Indoprofen (IC_{50} 4.6 μ M) also produced monophasic inhibition.

Figure 3 Concentration dependence for (A) inhibition of NAc-DNP-[³⁵S]Cys transport by naphthyl glucuronide and (B) the inhibition of naphthyl [¹⁴C]glucuronide transport by NAc-DNP-Cys in inside-out vesicles from human erythrocytes

The line drawn in (A) is the inhibition predicted from the Dixon equation for a two-component system in which 50% is inhibited by naphthyl glucuronide with an IC_{50} of 8.0 μ M and the remaining 50% is unaffected by naphthyl glucuronide; the line drawn in (B) is the inhibition predicted from the Dixon equation for a one-component system with an IC_{50} for NAc-DNP-Cys of 16 μ M. The substrate concentration was 5.0 μ M. The assay intervals at 37 °C were: NAc-DNP-[³⁵S]Cys, 2 min; naphthyl [¹⁴C]glucuronide, 5 min. The control transport rates were: NAc-DNP-[³⁵S]Cys, 62 pmol/min per mg of protein; and naphthyl [¹⁴C]glucuronide, 92 pmol/min per mg of protein. The ATP concentration was 1.0 mM.

Figure 4 Inhibition of naphthyl glucuronide-insensitive NAc-DNP-[³⁵S]Cys transport by indoprofen in inside-out vesicles from human erythrocytes

Transport at 5.0 μ M NAc-DNP-[³⁵S]Cys was measured in the presence of 100 μ M naphthyl glucuronide and various concentrations of indoprofen up to 300 μ M. The inset is a double-reciprocal plot of NAc-DNP-[³⁵S]Cys transport at various substrate concentrations; v is transport velocity (pmol/min per mg of protein), and s is NAc-DNP-[³⁵S]Cys concentration. The assay interval was 2 min at 37 °C. The ATP concentration was 1.0 mM.

ATP-dependent transport of NAc-DNP-[³⁵S]Cys in human erythrocyte vesicles showed a pronounced biphasic response to naphthyl glucuronide (Figure 3A). A Dixon plot analysis of the data fitted a two-component system in which the inhibitor-sensitive portion represented 50% of total transport and had an

Table 2 Kinetic constants for transport via MOAT4

K_m and V_{max} values for transported substrates were measured at 1.0 mM ATP, whereas K_m values for ATP were measured at 5.0 μ M naphthyl [¹⁴C]glucuronide, NAc-DNP-[³⁵S]Cys and [³H]DNP-SG, and 40 μ M [³H]methotrexate. Assay intervals at 37 °C were: NAc-DNP-[³⁵S]Cys, 2 min; naphthyl [¹⁴C]glucuronide, 5 min; [³H]DNP-SG and [³H]methotrexate, 10 min. Abbreviation: n.d., not determined.

Substrate	K_m (μ M)	V_{max} (pmol/min per mg)	K_m (ATP) (μ M)
mMOAT4			
Naphthyl glucuronide	10.5	120	270
NAc-DNP-Cys	6.0	78	250
Methotrexate*	170	27	230
DNP-SG†	450	148	250
hMOAT4			
Naphthyl glucuronide	6.4	305	185
NAc-DNP-Cys	10	150	n.d.

* From [31].

† From [20].

Figure 5 Double-reciprocal plot of naphthyl [¹⁴C]glucuronide transport with increasing substrate concentration in inside-out vesicles from L1210 cells

Additions were: BDT, bilirubin ditaurate, 2.0 μ M; EG, oestradiol 17-glucuronide, 10 μ M. v is transport velocity in pmol/min per mg of protein. The assay interval was 5 min at 37 °C. The ATP concentration was 1.0 mM.

IC_{50} for naphthyl glucuronide of 8 μ M. The remaining glucuronide-insensitive transport was sensitive to indoprofen (IC_{50} 16 μ M) (Figure 4), and a Dixon plot of the latter data was linear, indicating that only a single component was present.

ATP-dependent transport of naphthyl [¹⁴C]glucuronide in human erythrocyte vesicles was sensitive to NAc-DNP-Cys (Figure 3B), and the kinetics fitted a single component with an IC_{50} for NAc-DNP-Cys of 16 μ M.

Kinetic analysis of transport of NAc-DNP-Cys and naphthyl glucuronide

Kinetic constants for transport of NAc-DNP-[³⁵S]Cys and naphthyl [¹⁴C]glucuronide were determined from measurements of transport at varying substrate concentrations (Table 2). In L1210 vesicles, double-reciprocal plots of naphthyl [¹⁴C]glucuronide transport at substrate concentrations between 2 and

Figure 6 Double-reciprocal plot of NAc-DNP-[³⁵S]Cys transport with increasing substrate concentration in inside-out vesicles from L1210 cells

The concentration of naphthyl glucuronide was 30 μ M, and that of BPAA was 15 μ M. v is transport velocity in pmol/min per mg of protein. The assay interval was 2 min at 37 °C. The ATP concentration was 1.0 mM.

Figure 7 Double-reciprocal plot of naphthyl [¹⁴C]glucuronide transport with increasing substrate concentration in inside-out vesicles from human erythrocytes

Additions were: NAc-DNP-Cys, 30 μ M; tauroolithocholate 3-sulphate (TLCS), 3.0 μ M. v is transport velocity in pmol/min per mg of protein. The assay interval was 5 min at 37 °C. The ATP concentration was 1.0 mM.

50 μ M were linear (Figure 5) and yielded a K_m for half-maximal transport of 10.5 μ M and a V_{max} of 120 pmol/min per mg of protein. NAc-DNP-[³⁵S]Cys transport in L1210 vesicles also produced a linear double-reciprocal plot (Figure 6) with a K_m of 6.0 μ M and a V_{max} of 78 pmol/min per mg of protein, whereas transport of naphthyl [¹⁴C]glucuronide in human erythrocyte vesicles proceeded with a K_m of 6.4 μ M and a V_{max} of 305 pmol/min per mg of protein (Figure 7).

The kinetics of the glucuronide-insensitive transport component for NAc-DNP-[³⁵S]Cys in human erythrocyte vesicles was determined by performing transport measurements in the presence of 100 μ M naphthyl glucuronide. A linear double-

Table 3 Comparative responses of mMOAT4 to various inhibitors using [³H]DNP-SG, NAc-DNP-[³⁵S]Cys and naphthyl [¹⁴C]glucuronide as substrates

ATP-dependent transport [³H]DNP-SG (5.0 μ M) was measured (10 min at 37 °C) in L1210/C7 vesicles in the presence of 40 μ M EA-SG (see the legend to Table 1). Transport of 5.0 μ M NAc-DNP-[³⁵S]Cys (2 min at 37 °C) and 5.0 μ M naphthyl [¹⁴C]glucuronide (5 min at 37 °C) was measured in L1210 vesicles. K_i values were calculated from a Dixon plot of the reciprocal of transport with varying inhibitor concentration and the Dixon equation by using the K_m values shown in Table 2. Abbreviation: n.d., not determined.

Anion	K_i (μ M) for transport of:			Mean K_i (μ M)
	DNP-SG	NAc-DNP-Cys	Naphthyl glucuronide	
Bilirubin ditaurate	1.2	1.0	1.0	1.1
Indoprofen	3.0	2.2	3.1	2.8
NAc-DNP-Cys	5.0	5.5	6.1	5.5
Flurbiprofen	6.0	5.0	6.8	5.9
BPAA	5.5	7.7	8.5	7.2
4-Acridine glucuronide	n.d.	7.4	8.5	8.0
Naphthyl glucuronide	8.5	9.1	10.9	9.5
NAc-BSP-Cys	n.d.	10.9	11.5	11.2
Oestradiol-17-glucuronide	n.d.	14	13	13.5

reciprocal plot of transport as a function of substrate concentration was obtained (Figure 4, inset), and provided evidence for a transport system with a K_m for NAc-DNP-[³⁵S]Cys of 33 μ M and a V_{max} of 355 pmol/min per mg of protein.

Transport of NAc-DNP-[³⁵S]Cys by erythrocyte vesicles was also measured at various substrate concentrations in the absence of naphthyl glucuronide. The resulting double-reciprocal plot was non-linear, but a computer analysis of the data gave a close fit to a two-component system. When glucuronide-insensitive activity was assigned a K_m of 33 μ M and a V_{max} of 355 pmol/min per mg of protein, the remaining activity for NAc-DNP-[³⁵S]Cys was determined as proceeding with a K_m of 10 μ M and a V_{max} of 150 pmol/min per mg of protein (Table 2).

The close correlation between K_i values for inhibition of the low-affinity route for DNP-SG by naphthyl glucuronide and NAc-DNP-Cys (Table 1) and K_m values for the transport of NAc-DNP-[³⁵S]Cys and naphthyl [¹⁴C]glucuronide (Table 2) indicated that the same mMOAT4 carrier was mediating the transport of these three conjugates. Moreover, similar transport activities for NAc-DNP-[³⁵S]Cys and naphthyl [¹⁴C]glucuronide (Figure 2) suggested that human erythrocytes contain the human counterpart to mouse MOAT4.

Inhibitor sensitivity of mMOAT4 in L1210 vesicles with DNP-SG, NAc-DNP-Cys and naphthyl glucuronide as the transport substrate

The identification of mMOAT4 as a common transport system for [³H]DNP-SG, NAc-DNP-[³⁵S]Cys and naphthyl [¹⁴C]glucuronide was investigated further by comparing the effects of various anions, mercapturates and glucuronides on the transport of these substrates (Table 3). The results show that K_i values for the inhibition of [³H]DNP-SG, NAc-DNP-[³⁵S]Cys and naphthyl [¹⁴C]glucuronide transport were comparable for each of the inhibitors tested.

Sensitivity of MOAT4 to mercapturates and glucuronides

The specificity of mMOAT4 for various mercapturates and glucuronides was extended by using naphthyl [¹⁴C]glucuronide as the substrate (Table 4). The most potent inhibition by mercapturates was observed with *N*-acetyl leukotriene E₄ (NAc-LTE₄),

Table 4 Sensitivity of mouse and human MOAT4 to various mercapturates, glucuronides and bile acid conjugates with naphthyl [^{14}C]glucuronide as the substrate

ATP-dependent transport (5 min at 37 °C) was measured at 5.0 μM naphthyl [^3H]glucuronide with various concentrations of the indicated inhibitors in mouse L1210 or human erythrocyte vesicles. K_i values were calculated from the Dixon plot of the data and the Dixon equation by using the K_m values for naphthyl glucuronide shown in Table 2.

Inhibitor	K_i (μM) for naphthyl [^{14}C]glucuronide transport by:	
	mMOAT4	hMOAT4
Mercapturates		
NAC-DNP-Cys	6.1	8.4
NAC-LTE ₄	8.8	7.3
NAC-BSP-Cys	11.5	4.5
NAC-EA-Cys	210	215
Glucuronides		
Phenyl glucuronide	> 400	> 400
<i>p</i> -Nitrophenyl glucuronide	> 400	> 400
Naphthyl glucuronide	10.9	5.6
4-Acridine glucuronide	8.5	3.1
Oestradiol 17-glucuronide	13	13
Oestradiol 3-glucuronide	82	51
Oestradiol 3-glucuronide 17-SO ₄	68	24
Androsterone 3-glucuronide	145	104
Bile acid conjugates		
Taurocholate	270	370
Taurolithocholate	26	10.7
Taurolithocholate 3-SO ₄	6.5	2.0
Glycolithocholate 3-SO ₄	5.1	1.4

NAC-DNP-Cys and NAC-BSP-Cys, whereas the inhibition by the mercapturate of ethacrynic acid was surprisingly weak. Phenyl glucuronide and *p*-nitrophenyl glucuronide were poor inhibitors, whereas the slightly larger naphthyl glucuronide and 4-acridine glucuronide were bound with the highest affinity among the various glucuronides tested. Substantial selectivity was also seen for steroid glucuronides. Oestradiol 17-glucuronide exhibited a moderate affinity for mMOAT3 (K_i 13.5 μM), but affinity decreased to approx. one-fifth for both oestradiol 3-glucuronide and oestradiol 3-glucuronide 17-sulphate. Androsterone 3-glucuronide was the least effective inhibitor among the steroid glucuronides tested.

The corresponding hMOAT4 activity in human erythrocytes showed an inhibitor specificity that was strikingly similar to mMOAT4 (Table 4). Correlations were observed both in the magnitudes of K_i values and in relative affinities between groups of related compounds. The only pattern of variance was seen as a modest increase in affinity (2–4-fold) by hMOAT4 for certain representatives of different anion types, including monovalent anions (4-acridine glucuronide and taurolithocholate), divalent anions (oestradiol 3-glucuronide 17-sulphate, taurolithocholate 3-sulphate and glycolithocholate 3-sulphate) and a multivalent anion (NAC-BSP-Cys). Weak inhibition of hMOAT4 activity was observed by DNP-SG (K_i 1.4 mM). Daunorubicin and vincristine (at concentrations up to 250 μM) inhibited the transport of 5.0 μM naphthyl [^{14}C]glucuronide by less than 50 % in vesicles from either L1210 cells or human erythrocytes.

Kinetic studies of MOAT4 were also performed to determine the type of inhibition produced by selected anions and anion conjugates. Double-reciprocal plots of transport at various substrate concentrations were performed at constant inhibitor levels, and y -intercepts relative to the control without inhibitor

were determined. Competitive inhibition was indicated by a common y -intercept for each of the combinations tested. Transport of naphthyl [^{14}C]glucuronide by mMOAT4 (Figure 5) was inhibited competitively by bilirubin ditaurate and oestradiol 17-glucuronide; transport of NAc-DNP-[^{35}S]Cys by mMOAT4 (Figure 6) was inhibited competitively by naphthyl glucuronide and BPAA; and transport of naphthyl [^{14}C]glucuronide by hMOAT4 (Figure 7) was inhibited competitively by NAc-DNP-Cys and taurolithocholate 3-sulphate.

Inhibitor sensitivity of the glucuronide-insensitive portion of NAc-DNP-Cys transport in human erythrocyte vesicles

The glucuronide-insensitive portion of NAc-DNP-Cys transport in human erythrocyte vesicles was evaluated for inhibitor sensitivity by using NAc-DNP-[^{35}S]Cys as the substrate and 100 μM naphthyl glucuronide to block hMOAT4 activity. IC_{50} values were obtained from transport measurements at various inhibitor concentrations, and converted into a K_i by using the Dixon equation and a K_m for NAc-DNP-[^{35}S]Cys of 33 μM . DNP-SG (200 μM), EA-SG (200 μM) and NAc-LTE₄ (20 μM) had no effect on NAc-DNP-[^{35}S]Cys transport, but inhibition was seen with other selected monovalent and divalent anions, including bilirubin ditaurate (K_i 2.2 μM), indoprofen (K_i 15 μM), taurolithocholate 3-sulphate (K_i 18 μM), BSP (K_i 24 μM) and flurbiprofen (K_i 170 μM).

Michaelis constants for ATP

The dependence of transport on the concentration of ATP was measured for mMOAT4 with NAc-DNP-[^{35}S]Cys and naphthyl [^{14}C]glucuronide as the substrate. Double-reciprocal plots of transport with increasing concentrations of ATP were linear for each substrate up to 2.0 mM ATP; however, higher levels were inhibitory and produced non-linear kinetics indicative of substrate inhibition. The K_m for ATP was 270 μM with naphthyl [^{14}C]glucuronide as the substrate and 250 μM with NAc-DNP-[^{35}S]Cys as the substrate (see Table 2). hMOAT4 exhibited a K_m for ATP of 185 μM . Glucuronide-insensitive transport of NAc-DNP-[^{35}S]Cys in human erythrocyte vesicles was characterized by a K_m for ATP of 350 μM . The latter was determined from transport at 5.0 μM NAc-DNP-[^{35}S]Cys (2 min, 37 °C) in the presence of 100 μM naphthyl glucuronide and various concentrations of ATP.

DISCUSSION

DNP-SG, leukotriene C₄ and other GS-conjugates are transported out of cells via unidirectional ATP-dependent efflux systems whose characteristics have been determined by using inside-out vesicles from various cell sources. Although described initially as a GS-X pump [2] to coincide with a broad specificity for GS-conjugates, an alternative designation as a MOAT had been adopted more recently to reflect the likely inclusion of a more diverse range of substrates, such as dianionic conjugates [11,26,27]. However, it has become evident that several genetically distinct systems mediate the efflux of organic anions and anion conjugates. Two of these transporters, cMOAT and MRP, have been shown to be separate entities by differences in amino acid sequence [28]. cMOAT, the canalicular efflux pump of rat liver, was also differentiated from the GS-X pump of rat erythrocytes by studies in the TR⁻ rat [24], and a variant L1210 cell line with a defective high-affinity efflux system for DNP-SG and a normal low-affinity system provided genetic evidence for differences

between these two routes [20]. Hence the number of different MOAT exporters has been expanding but a standard terminology for these ATP-dependent transport systems has not been developed. One possible solution is to employ the MOAT designation with a numerical extension to identify different representatives of the MOAT family. Thus MOAT1 could be employed as an alternative term for cMOAT, and rMOAT1 could refer to the cMOAT of rat liver. Human MRP could be designated hMOAT2. Alternative terminology in which a numerical extension is appended to MRP (MRP-1, MRP-2, etc.) was not employed because some systems within this group might not be associated with the development of multidrug resistance, whereas the multispecific transport of anionic compounds is, so far, a common trait of each system. Tissue designations for sites of activity is confusing because transporters such as MRP occur in many different cells and tissues [30].

The high-affinity GS-conjugate pump of L1210 cells can be distinguished from the rat canalicular GS-conjugate system (rMOAT1) and MRP (hMOAT2) on the basis of differences in specificity. Various findings suggest that cMOAT and MRP differ from the GS-X pump of L1210 cells by the ability to transport glucuronides. In rat canalicular membranes, the transport of *p*-nitrophenyl glucuronide [12] and nafenopin glucuronide [14] are each sensitive to low levels of DNP-SG, a substrate of cMOAT, and DNP-SG transport is inhibited by naphthyl glucuronide [37]. Biliary excretion of various glucuronides [11], including naphthyl glucuronide [38], is also defective in the TR⁻rat. Similarly, MRP has been shown to transport oestradiol 17-glucuronide (K_m 1.5 μ M) and also the monoglucuronide of etoposide [15]. In contrast, the GS-X pump for DNP-SG in L1210 cells is unable to transport naphthyl glucuronide and is not inhibited by *p*-nitrophenyl glucuronide (Table 1), naphthyl glucuronide (Table 1) or oestradiol glucuronide (results not shown). These kinetic differences suggest that the high-affinity transporter for DNP-SG in L1210 cells is most likely to reside in a separate MOAT category, probably in the MOAT3 classification along with the erythrocyte GS-X pump. Information at the molecular level, however, will be needed to verify that the GS-X pump activities of erythrocytes and L1210 cells are comparable transporters and represent gene products different from cMOAT and MRP.

L1210 cells contain an ATP-dependent efflux system for glucuronides and mercapturates that can be distinguished from MOAT1, MOAT2 and MOAT3 by a very low affinity for leukotriene C₄, DNP-SG and various other GS-conjugates [20]. This separate activity, designated in L1210 mouse cells as mMOAT4, mediates the transport of both NAc-DNP-Cys and naphthyl glucuronide, as determined by similar K_m and K_i values for these two substrates (Tables 2–4), and by a close correlation in the affinity and order of effectiveness for inhibition by various anions and anion conjugates (Table 4). Human erythrocytes contain a comparable hMOAT4 transport activity on the basis of substantial similarities in inhibitor specificity (Table 4). Direct measurements established that MOAT4 is the only detectable route for the transport of naphthyl glucuronide in both L1210 cells and human erythrocytes, and inhibition of naphthyl glucuronide transport by various other glucuronides indicates that this route is involved in the general uptake of glucuronides. The mouse and human systems can be distinguished because the latter has a higher V_{max} for transport of both naphthyl glucuronide and NAc-DNP-Cys and has a modestly increased affinity for several anion conjugates (Table 4). Naphthyl [¹⁴C]glucuronide serves as a judicious substrate for measuring MOAT4 from either source owing to the lack of interfering activities and a high substrate affinity.

mMOAT4 is the same ATP-dependent activity that had been shown previously in L1210 cells to mediate the low-affinity efflux of DNP-SG [20]. mMOAT4 is characterized by a high affinity for NAc-DNP-Cys, naphthyl glucuronide and a number of other monovalent and divalent anions (Tables 1–4). Moreover, competitive inhibition by various anions (Figures 5–7) suggests that substrates for mMOAT4 extend to a wide range of mercapturates, glucuronides and diconjugates of bilirubin and bile acids. Previous studies had also shown that the same mMOAT4 activity in L1210 cells mediates the transport of methotrexate [31]. The broad binding capacity for various anions and anionic conjugates is also accompanied by some binding restraints. A critical size is required, because naphthyl glucuronide acts as a high-affinity substrate, whereas the slightly smaller *p*-nitrophenyl glucuronide and phenyl glucuronide interact poorly, or not at all, with this system (Table 1). Considerations other than a large size also impact on specificity because NAc-DNP-Cys is smaller than the mercapturate of ethacrynic acid yet exhibits a 20-fold higher affinity. The conjugation position of otherwise equivalent isomers can also provide substantial binding differences because transposing the site of glucuronide attachment in oestradiol from the 17 position to the 3 position causes a decrease in affinity to one-sixth (Table 4).

A low-affinity transport activity for DNP-SG has been described previously in human erythrocytes [17,18], and information was reported on substrate specificity. Bartosz et al. [18] showed that transport at 2 mM DNP-SG after 60 min was inhibited by various anions and anion conjugates, including bilirubin ditaurate, tauroolithocholate 3-sulphate and oestradiol 17-glucuronide, but the reported affinities for these compounds were one-tenth of those observed in the present study for hMOAT4 with a 5 min incubation and naphthyl [¹⁴C]glucuronide as the substrate. Moreover, the same study reported affinities for naphthyl glucuronide and NAc-DNP-Cys that were one-fiftieth to one-hundredth of those observed here. These variations could have resulted from measurements of the same transporter under substantially different assay conditions, or might reflect the properties of a separate transport activity at very high levels of DNP-SG.

Human erythrocytes express an additional ATP-dependent transport activity for NAc-DNP-[³⁵S]Cys that is insensitive to naphthyl glucuronide. The latter activity contributed 50 % of total transport at 5.0 μ M NAc-DNP-[³⁵S]Cys (Figure 3A), and was deduced to be a single component by its monophasic response to indoprofen (Figure 4) and by a linear double-reciprocal plot of transport with varying substrate concentration (inset, Figure 4). The accompanying activity was identified as hMOAT4 by its sensitivity to naphthyl glucuronide (K_i 5.3 μ M) and by its K_m of 10 μ M for NAc-DNP-Cys transport. L1210 cells might also express low levels of the same glucuronide-insensitive transporter because a small portion (4 %) of NAc-DNP-Cys transport in L1210 vesicles was insensitive to naphthyl glucuronide (Figure 2A). This transporter could be distinguished kinetically from MOAT1, MOAT2 and MOAT3 by its insensitivity to GS-conjugates (EA-SG and DNP-SG), and from MOAT4 by its insensitivity to naphthyl glucuronide and NAc-LTE₄; it may therefore represent a separate member of the MOAT family of transporters. The high affinity for bilirubin ditaurate (K_i 2.2 μ M) suggests that divalent anion conjugates could be the primary substrates for this glucuronide-insensitive transport system.

Hydrophobic xenobiotics, electrophiles and endogenous compounds are metabolized to a variety of possible structures. Many of these compounds are metabolized by glutathione S-transferases [39,40] and UDP-glucuronyl transferases [41], and the result can be a range of structurally diverse products. The further

metabolism of GS-conjugates gives rise to various mercapturates. The detoxification process is completed by the extrusion of anionic and zwitterionic conjugates of various sizes and structures via efflux pumps. The capacity for handling a diverse range of conjugates arises from the presence of multiple efflux routes with varying and overlapping specificities and enables cells to maintain considerable flexibility in extruding both endobiotic and xenobiotic anions and conjugates. In human erythrocytes and L1210 cells, at least two multispecific organic anion transporters (MOAT3 and MOAT4) are present that have the combined capacity to mediate the efflux of GS-conjugates, large monovalent and divalent anions, glucuronides and mercapturates.

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