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ATP-dependent Transport of Leukotrienes B₄ and C₄ by the Multidrug Resistance Protein ABCC4 (MRP4)

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Abbreviations: ABCC, ATP-binding cassette transporter, subfamily C; C-tau, cholyltaurine; DTT, dithiotreitol; GSH, reduced glutathione; LT, leukotriene; MRP, multidrug resistance protein; PG, prostaglandin; PMN, human neutrophilic polymorphonuclear leukocyte.

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Abstract

The proinflammatory mediators leukotriene (LT) B₄ and LTC₄ must be transported out of cells before they can interact with LT receptors. Previously, we identified the multidrug resistance protein ABCC1 (MRP1) as an efflux pump for LTC₄. However, the molecular basis for the efflux of LTB₄ was unknown. Here we demonstrate that human ABCC4 mediates the ATP-dependent efflux of LTB₄ in the presence of reduced glutathione (GSH), whereby the latter can be replaced by S-methyl GSH. Transport studies were performed with inside-out membrane vesicles from V79 fibroblasts and Sf9 insect cells that contained recombinant ABCC4, with vesicles from human platelets and myelomonocytic U937 cells, which were rich in endogenous ABCC4 whereas ABCC1 was below detectability. Moreover, human polymorphonuclear leukocytes contained ABCC4. Kₘ values for LTB₄ were 5.2 µM with vesicles from fibroblasts and 5.6 µM with vesicles from platelets. ABCC4, with its broad substrate specificity, also functioned as an ATP-dependent efflux pump for LTC₄ with a Kₘ of 0.13 µM in vesicles from fibroblasts and 0.32 µM in vesicles from platelets. However, GSH was not required for the transport of this glutathionylated leukotriene. The transport of LTC₄ by ABCC4 explains its release from platelets during transcellular synthesis. ATP-dependent transport of LTB₄ and LTC₄ by ABCC4 was inhibited by several organic anions, including S-decyl GSH, sulindac sulfide, and by the LTD₄ receptor antagonists montelukast and MK571. Thus, as an efflux pump for the proinflammatory mediators LTB₄ and LTC₄, ABCC4 may represent a novel target for anti-inflammatory therapies.
Introduction

Leukotrienes (LTs) are biologically potent proinflammatory lipid mediators mainly derived from arachidonate (for reviews see Samuelsson et al., 1987; Funk, 2001; Folco and Murphy, 2006). The biosynthesis of LTB\textsubscript{4} and of LTC\textsubscript{4} is followed by a distinct export step recognized originally in human leukocytes (Lam et al., 1989; Lam et al., 1990), murine mastocytoma cells (Schaub et al., 1991), and plasma membrane vesicles from normal and transport-deficient rat hepatocytes (Keppler, 1992). Efflux transport of LTB\textsubscript{4} and LTC\textsubscript{4} is an essential step in cells containing all enzymes of LT biosynthesis (for review see Funk, 2001) and also in transcellular LT biosynthesis (reviewed by Folco and Murphy, 2006). Thus, LTA\textsubscript{4} taken up from other cells is actively converted to LTC\textsubscript{4} in platelets and effluxed by an energy-dependent export pump (Maclouf and Murphy, 1988; Sjölinder et al., 1999). Moreover, erythrocytes converted exogenous LTA\textsubscript{4} to LTB\textsubscript{4} which was subsequently exported into the extracellular space (Fitzpatrick et al., 1984).

The multidrug resistance protein 1 (MRP1), now termed ATP-binding cassette transporter, subfamily C, member 1 (ABCC1) was the first ATP-dependent export pump identified for LTC\textsubscript{4} (Jedlitschky et al., 1994; Leier et al., 1994a). The link between ABCC1 and LTC\textsubscript{4} as its substrate was established primarily by photoaffinity labeling studies using LTC\textsubscript{4} with its conjugated triene structure as a high-affinity ligand and the LTD\textsubscript{4} receptor antagonist MK571 as a competitive inhibitor of photoaffinity labeling and transport (Leier et al., 1994b; Jedlitschky et al., 1994; Leier et al., 1994a). However, ABCC1 does not transport LTB\textsubscript{4} (see below). Thus, the molecular identity of the biologically important efflux transporter for LTB\textsubscript{4} has been unknown.

The members of the multidrug resistance protein subfamily, including ABCC1-6 and ABCC10-12, have a rather broad substrate specificity and mediate the ATP-dependent efflux of organic anions, including glutathione conjugates such as LTC\textsubscript{4}, across the plasma membrane into the extracellular space (for review see Deeley et al., 2006). In the case of
ABCC1 (Loe et al., 1996) and ABCC4 (MRP4; Rius et al., 2003; Rius et al., 2006), it was recognized, however, that the presence or the co-transport of reduced glutathione (GSH) may change the substrate specificity and allow the efflux of substances that are not transported in the absence of GSH. GSH occurs in living cells at millimolar concentrations (Meister, 1988), and modulation of the substrate specificity of ABCC transporters by GSH represents a physiological condition. For example, we showed that ABCC4 transports most bile acids only in the presence of GSH (Rius et al., 2003; Rius et al., 2006).

These insights prompted us to study the ATP-dependent transport of LTB₄ and LTC₄ by ABCC4 in the presence or absence of GSH. ABCC4 is widely distributed in cells and tissues, including prostate (Lee et al., 2000), urogenital tissues (Rius et al., 2005), kidney proximal tubules (van Aubel et al., 2002), astrocytes and capillary endothelial cells of the brain (Nies et al., 2004), platelets (Jedlitschky et al., 2004), erythrocytes (Klokouzas et al., 2003), hepatocytes (Rius et al., 2003), many cancer cells lines (Szakacs et al., 2004), and, as shown in this contribution, in human neutrophilic polymorphonuclear leukocytes. Here we report our studies of the role of ABCC4 in the ATP-dependent efflux transport of LTB₄ and LTC₄ from cells. For this aim, we decided to use several cell lines stably expressing recombinant human ABCC4 (V79-ABCC4, HEK-ABCC4, and Sf9-ABCC4), as well as human platelets, HeLa, and U937 cells, which are remarkably rich in endogenous ABCC4. This approach was taken because of the widespread occurrence of endogenous ABCC4/Abcc4 in cell lines used for transfection and because of the lack of human cell lines deficient in ABCC4. Otherwise, knock-out mice lacking Abcc4 expression, although a valuable animal model, do not provide a convincing answer to the question of the ATP-dependent transporter in the plasma membrane responsible for the release of LTB₄ because of major kinetic differences shown for other substrates between murine Abcc4 and human ABCC4 (Wolf de et al., 2007) and because of a likely compensatory adaptation by up-regulation of alternative efflux pumps.
Methods

**Materials.** \([5,6,8,9,11,12,14,15-^3\text{H}]\text{Leukotriene B}_4\) (LTB\(_4\); 165 Ci/mmol), \([14,15,19,20-^3\text{H}]\text{leukotriene C}_4\) (LTC\(_4\); 158 Ci/mmol), \([14,15,19,20-^3\text{H}]\text{leukotriene D}_4\) (LTD\(_4\); 167 Ci/mmol), and \(^{3}\text{H}\)-cholyltaurine (C-tau; 1.19 Ci/mmol) were purchased from Perkin Elmer (Boston, MA). LTC\(_4\) and LTD\(_4\) were from Cayman Chemical (Ann Arbor, MI) and MK571 was from Alexis Biochemicals (Lausen, Switzerland). Montelukast was purchased from Sequoia Research Products (Pangbourne, United Kingdom). 5-Hydroxy-6,8,11,14-eicosatetraenoate was from Biomol International (Plymouth Meeting, PA) and Sephadex G-50 fine from Amersham Bioscience (Uppsala, Sweden). LTB\(_4\), GSH, S-methyl GSH, S-decyl GSH, dithiothreitol (DTT), 12-hydroxy-5,8,10,14-eicosatetraenoate, prostaglandin E\(_2\) (PGE\(_2\)), C-tau, dipyridamole, sulindac sulfide, indomethacin, probenecid, and sulfipyrazone were obtained from Sigma (St. Louis, MO). Isolated membranes from Sf9 cells containing ABCC4 (Sf9-ABCC4) and Sf9 control cells (Sf9-Co) were purchased from Solvo Biotechnology (Budapest, Hungary).

**Antibodies.** The SNG antiserum was raised against the carboxy terminus of human ABCC4 sequence (SNGQPSTLTIFETAL; Rius et al., 2003). The protein affinity–purified SNG antiserum was obtained as described (Rius et al., 2003). The monoclonal antibody M4I-10 against ABCC4 was from Alexis Biochemicals and the monoclonal antibody QCRL-1 against ABCC1 was from Biozol (Eching, Germany). The horseradish peroxidase–conjugated goat anti-rabbit, anti-rat, and anti-mouse IgGs were from Bio-Rad (Munich, Germany).

**Cell lines and cell culture.** Chinese hamster lung V79 fibroblasts permanently expressing human recombinant ABCC4 (V79-ABCC4) were grown in Dulbecco’s modified Eagle’s medium (Sigma), supplemented with 10% (v/v) fetal bovine serum and 100 units/ml
penicillin/streptomycin, and kept at 37 °C and 5% CO₂, as described (Rius et al., 2003).

Human embryonic kidney HEK293 cells were grown in minimum essential medium (Sigma), supplemented with 10% (v/v) fetal bovine serum and 100 units/ml penicillin/streptomycin, and kept at 37 °C and 5% CO₂. HEK293 cells were transfected with the pREP9-ABCC4 cDNA construct or the vector only (Rius et al., 2003), using FuGENE 6 transfection reagent (Roche Diagnostics, Mannheim, Germany). After 48 h, the cells were split, and stable transfectants were selected using medium with 1 mg/ml G418 (Gibco, Karlsruhe, Germany). Resistant clones were screened by immunoblot analysis and immunofluorescence microscopy for ABCC4 expression as described (Rius et al., 2003). Sodium butyrate (5 mM) was added to the cells 24 h before harvesting to enhance the expression of the recombinant protein (Cui et al., 1999). The non-P-glycoprotein-expressing doxorubicin-resistant HL60-ADR cells (Marquardt et al., 1990; Krishnamachary and Center, 1993) were grown in RPMI medium (Sigma), supplemented with 10% (v/v) fetal bovine serum, 100 units/ml penicillin/streptomycin, and 200 nM daunorubicin, and kept at 37 °C and 5% CO₂ (Jedlitschky et al., 1994). The vector control transfected HeLa cells were grown in RPMI medium, supplemented with 10% (v/v) fetal bovine serum and 100 units/ml penicillin/streptomycin (Grant et al., 1994). The parental U937 cells, a human myelomonocytic leukemia cell line, were obtained from the American Type Culture Collection (Manassas, VA) and were cultured in RPMI medium, supplemented with 10% (v/v) fetal bovine serum and 100 units/ml penicillin/streptomycin.

**Preparation of membrane vesicles from human platelets.** Plasma membrane fractions from human platelets were isolated as described (Broekman, 1992; Jedlitschky et al., 2004). Briefly, platelet-rich plasma was obtained from the Blood Center of the University of Heidelberg hospitals. The platelets were disrupted in homogenization buffer (100 mM KCl, 25 mM NaCl, 2 mM MgSO₄, 12 mM Na₃ citrate, 10 mM D-glucose, 25 mM Hepes, 5
mM ATP, 0.35% BSA, pH 7.0) by freezing in liquid nitrogen and thawing at 37 °C four times. The homogenate was layered onto a linear 30–60 % sucrose density gradient. After ultracentrifugation at 200,000 x g for 60 min at 4 ºC, 4 fractions were collected corresponding to plasma membrane (30 % sucrose), lysosomes (35-40 % sucrose), α-granules (50-55 % sucrose), or dense granules (60 % sucrose). They were further homogenized and washed by centrifugation at 100,000 x g in incubation buffer (250 mM sucrose, 10 mM Tris/HCl, pH 7.4), and passed 20 times through a 27-gauge needle for vesicle formation. Aliquots of the membrane vesicle suspension were stored in liquid nitrogen.

**Preparation of membrane vesicles from human erythrocytes.** Inside-out plasma membrane vesicles from human erythrocytes were prepared as described (Steck and Kant, 1974). Briefly, human blood was obtained from a normal healthy donor and collected into EDTA syringes. Erythrocytes were washed three times by centrifugation at 2,300 x g for 10 min at 4 ºC, discarding the buffy coat each time from the surface of the pellet. Hemolysis was achieved in 5P8 buffer (5 mM sodium phosphate, pH 8.0), followed by centrifugation at 25,000 x g for 10 min at 4 ºC. The resulting membranous ghosts were suspended in 0.5P8 buffer (1:10 dilution of 5P8 buffer) and slowly mixed for 3 h on ice. After centrifugation at 25,000 x g (10 min at 4 ºC), the membranes were washed by centrifugation at 40,000 x g (30 min at 4 ºC) in 0.5P8 buffer. The membranes were resuspended in incubation buffer (250 mM sucrose, 10 mM Tris/HCl, pH 7.4) and passed 10 times through a 27-gauge needle for vesicle formation. Aliquots of the membrane vesicle suspension were frozen and stored in liquid nitrogen.

**Preparation of membrane vesicles from mammalian cell lines.** Inside-out plasma membrane vesicles from transfected V79 and HEK293 cells, HL60-ADR, control HeLa, and parental U937 cells were prepared as described (Keppler et al., 1998). Briefly, the cells were
lysed by incubation in hypotonic buffer (0.1 mM EDTA, 0.5 mM sodium phosphate, pH 7.0) for 1.5 h, followed by homogenization with a Potter-Elvehjem homogenizer. After centrifugation of the homogenate at 12,000 x g (10 min at 4 °C), the postnuclear supernatant was centrifuged at 100,000 x g for 45 min at 4 °C. The resulting pellet was suspended in incubation buffer (250 mM sucrose, 10 mM Tris/HCl, pH 7.4), homogenized with a tight-fitting Dounce (glass/glass) homogenizer, and layered over 38 % sucrose in 5 mM HEPES/KOH, pH 7.4. After centrifugation at 280,000 x g for 1.5 h at 4 °C, the turbid layer at the interface was collected, washed by centrifugation in the incubation buffer (100,000 x g), and passed 20 times through a 27-gauge needle for vesicle formation. Aliquots of the membrane vesicle suspension were frozen and stored in liquid nitrogen.

Isolation of human PMNs. Human blood was obtained from a normal healthy donor and collected into EDTA-containing syringes. PMNs were isolated using a single-step gradient (Polymorphprep, Axis-Shield, Oslo, Norway) according to the manufacturer's instructions. Contaminating erythrocytes were lysed by hypotonic shock. The purity of PMNs was examined on Giemsa-stained smears indicating more than 97 % PMNs in the cell preparation.

Immunoblot analysis. Membrane vesicles (10–50 µg protein) from cell lines and human platelets and erythrocytes were diluted with sample buffer and incubated at 37 °C for 30 min before their separation on a 7.5 % SDS polyacrylamide gel. Immunoblotting was performed using a tank blotting system (Bio-Rad) and an enhanced chemiluminescence horseradish peroxidase detection system (Amersham Biosciences). The polyclonal SNG (Rius et al., 2003) antiserum was diluted 1:1000 in PBS containing 0.05 % Tween 20, the monoclonal M4I-10 antibody was diluted 1:50, and the protein affinity-purified SNG antiserum and the monoclonal QCRL-1 antibody were diluted 1:500. For peptide competition
studies, the SNG antiserum (1:1000 dilution) or the affinity-purified SNG antiserum (1:500 dilution) was incubated for 16 h at 4 °C with 10 µM of the synthetic peptide used to generate the SNG antiserum, and then applied onto the blots as the primary antibody. The horseradish peroxidase–conjugated goat anti-rabbit, anti-rat, and anti-mouse antibodies were used at dilutions of 1:2000, 1:5000, and 1:3000, respectively.

Confocal immunofluorescence microscopy of human PMNs and of cultured cells. Isolated human PMNs and U937 cells were fixed for 30 min in 4 % paraformaldehyde, permeabilized for 30 min in 1 % Triton X-100 and immunostained as described (Rius et al., 2003). The protein affinity-purified SNG antiserum was diluted 1:20 and the Alexa Fluor 488-conjugated goat anti-rabbit IgG was diluted 1:300. Confocal immunofluorescence pictures were taken on a LSM510 (Carl Zeiss, Oberkochen, Germany).

Vesicle transport studies. ATP-dependent transport of [3H]LTB4 into inside-out membrane vesicles was measured by centrifugation through Sephadex G-50 columns essentially as described (Keppler et al., 1998). This procedure is more efficient for the separation of extravesicular labeled substrates than the filtration through nitrocellulose filters, as labeled lipophilic substrates bound to the filters may cause high background radioactivity. Membrane vesicles were incubated at 37 °C with 4 mM ATP, 10 mM MgCl2, 10 mM creatine phosphate, 100 µg/ml creatine kinase, labeled substrate, in the presence or absence of 5 mM GSH, in incubation buffer containing 250 mM sucrose, and 10 mM Tris/HCl at pH 7.4. DTT (1 mM) was added to the incubation buffer for measurements in the presence and absence of GSH. The final incubation volume was 55 µl. The substrate and inhibitor concentrations are given in the respective figure legends and tables. For inhibition studies, compounds were added from a stock solution in an appropriate solvent (incubation buffer, ethanol, or dimethyl sulfoxide at a final concentration of the solvent below 0.5 % v/v) and identical concentrations
of the solvent were used in control samples. NICK spin columns (0.2 g of Sephadex G-50 per 3.3 ml of incubation buffer) were prepared by rinsing with incubation buffer and were centrifuged at 400 x g for 3 min at 4 °C immediately before use. Aliquots (15 or 20 µl) of the incubations were taken at the times indicated, diluted with ice-cold incubation buffer (final volume 100 µl), and immediately loaded onto the Sephadex G-50 columns. The columns were additionally rinsed with 100 µl of incubation buffer and centrifuged at 400 x g for 3 min at 4 °C. The effluents were collected, dissolved in liquid scintillation fluid, and counted for radioactivity. In control experiments, ATP was replaced by an equal concentration of the nonhydrolyzable analog β,γ-methylene-ATP (AMP-PCP). Rates of net ATP-dependent transport were calculated by subtracting values obtained in the presence of AMP-PCP as a blank from those in the presence of ATP.

ATP-dependent transport of [³H]LTC₄, [³H]LTD₄, and [³H]C-tau into inside-out membrane vesicles was measured by rapid filtration through nitrocellulose filters as described (Keppler et al., 1998). For blanks, ATP was replaced by 5′-AMP and aliquots of the incubations were filtered immediately through nitrocellulose filters (0.2-µm pore size, Millipore, Billerica, MA) pre-soaked in incubation buffer. Filters were rinsed with 8 ml of incubation buffer, dissolved in liquid scintillation fluid, and counted for radioactivity.

For determination of kinetic constants, transport rates were measured at five different substrate concentrations (0.1–1 µM for LTB₄, 1–10 mM for GSH, and 0.1–2 µM for LTC₄). Kₘ values were determined as the substrate concentration at half-maximal velocity of transport under these conditions by use of double-reciprocal plots and direct curve-fitting to the Michaelis-Menten equation.

For the statistical analysis, Student’s t-test was used. A P value < 0.01 was considered significant, and a P value < 0.001 was considered highly significant.
Results

**Cellular systems to study endogenous and recombinant ABCC4.** Several cellular preparations were examined for the endogenous expression of ABCC4 as possible tools for transport studies. Consistent with previous findings, the polyclonal SNG antiserum detected broad bands between 170 and 200 kDa in membrane vesicles from human platelets and erythrocytes (Fig. 1A, upper panel), as well as in tissue homogenates from prostate (Jedlitschky et al., 2004; Klokouzas et al., 2003; Lee et al., 2000; Rius et al., 2005). In erythrocytes, we detected an additional band at 120 kDa, which was probably the result of protein degradation. Strong signals were also detected in membrane vesicles from HL60-ADR, HeLa-Co, and U937 cells, indicating the endogenous expression of ABCC4 in these cell lines (Fig. 1A, upper panels). A similar pattern of bands was obtained with the monoclonal antibody M₄L-10 (Fig. 1A, middle panels) and the affinity-purified SNG antiserum (data not shown). The ABCC4-specific signal was abolished in all samples when the SNG or the affinity-purified SNG antisera were incubated with the synthetic SNG peptide before immunoblot analysis (data not shown). Cell-specific variations in the apparent molecular mass were likely due to differences of complex glycosylation in the different cell lines.

Because of the high levels of endogenous ABCC4 in the cell and tissue preparations (Fig. 1A, upper and middle panels), ABCC1 levels were analyzed under the same conditions to exclude cross-reactivity of the SNG antiserum (Fig. 1A, lower panels). The monoclonal QCRL-1 antibody detected a broad band between 170 and 190 kDa in vesicles from HL60-ADR cells, as well as a faint band at 200 kDa in vesicles from HeLa-Co cells (Fig. 1A, lower panel). However, with the identical protein amount of 10 µg and equal exposure time, no ABCC1 was detected in the plasma membrane vesicles from human platelets, erythrocytes, U937 cells, or in tissue homogenates from prostate (Fig. 1A, lower panels). ABCC1 protein
in human erythrocytes was detectable only after long exposure times or with relatively large protein amounts (data not shown).

Increasing amounts of membrane vesicles from human platelets were examined to compare the detection of ABCC4 and ABCC1 (Fig. 1B). The SNG antiserum and the monoclonal antibody M41-10 detected much greater signal intensity as the amount of protein increased (Fig. 1B, upper and middle panels), whereas the QCRL-1 antibody directed against ABCC1 detected no protein at all (Fig. 1B, lower panel). A weak signal for ABCC1 was obtained only when the exposure time of the blot was extended 20 times as long as in Fig. 1B (data not shown). These results indicate that human platelets, HeLa-Control and U937 cells are a rich source of endogenous ABCC4 and have, if any, extremely low levels of endogenous ABCC1 (Fig. 1A and B).

Cell lines expressing recombinant ABCC4 were also examined by immunoblotting (Fig. 1C). The SNG antiserum detected a broad band at 170 to 190 kDa in membrane vesicles from hamster V79 fibroblasts and HEK293 cells transfected with recombinant ABCC4 (Fig. 1C). The vesicles from the HEK293 cells transfected with the empty vector also showed a significant level of endogenous ABCC4 protein (Fig. 1C). Vesicles from the Sf9 insect cells containing recombinant human ABCC4 presented a narrow band at 150 kDa, consistent with expression in a cell system which lacks complex glycosylation (van Aubel et al., 2002).

Detection of ABCC4 in human PMNs and in parental U937 cells by confocal laser scanning microscopy. Expression of ABCC4 was analyzed in human PMNs (Fig. 1D) and in U937 cells (Fig. 1D) by immunofluorescence microscopy. Incubation of PMNs with the affinity-purified SNG antibody yielded strong fluorescent signals predominantly in intracellular membrane structures but also in the plasma membrane (Fig. 1D, upper panels). This staining pattern was abolished in human PMNs (Fig. 1D, lower left panel), when the affinity-purified SNG antibody was preincubated with the synthetic SNG peptide. In the
parental U937 cells, a human myelomonocytic cell line, ABCC4-specific staining revealed a similar pattern as for human PMNs (Fig. 1D, lower right panel). The immunolocalization of ABCC4 in U937 (Fig. 1D, lower right panel) confirms the endogenous ABCC4 expression detected by immunoblot analysis in this cell line (Fig. 1A, upper and middle panels).

ATP-dependent transport by ABCC4 of the standard substrate C-tau in the presence of GSH. Membrane vesicles containing endogenous or recombinant ABCC4 were assayed for transport activity using 5 µM [3H]C-tau in the presence or absence of 5 mM GSH during a 10-min period as described earlier (Rius et al., 2003). Among the four membrane fractions isolated from human platelets, the fraction containing mainly plasma membranes showed the highest stimulation of the ATP-dependent [3H]C-tau transport by GSH or S-methyl GSH, which typically indicated ABCC4 activity. Membrane vesicles from the platelet plasma membrane fraction showed an increase in ATP-dependent [3H]C-tau transport from 0.7 to 2.8 pmol x mg protein⁻¹ x min⁻¹ in the presence of GSH. Therefore, further transport studies were performed using the membrane vesicles from the plasma membrane fraction of human platelets. In membrane vesicles from HeLa-Co and U937 cells, ATP-dependent [3H]C-tau transport was also greatly increased by GSH (in HeLa-Co membrane vesicles, without GSH: 1.7; with GSH 14.3 pmol x mg protein⁻¹ x min⁻¹; in U937 membrane vesicles, without GSH: 0.6; with GSH 7.2 pmol x mg protein⁻¹ x min⁻¹).

Recombinant ABCC4 was also assayed for transport of C-tau in the presence of GSH and S-methyl GSH. Membrane vesicles from ABCC4 containing V79 cells, HEK-ABCC4, and Sf9-ABCC4 cells were highly active in GSH and S-methyl GSH-dependent C-tau transport with the following transport rates: in V79-ABCC4 membrane vesicles, without GSH: 3.9; with GSH 47.1 pmol x mg protein⁻¹ x min⁻¹; in HEK-ABCC4 membrane vesicles, without S-methyl GSH: 1.9; with S-methyl GSH 34.9 pmol x mg protein⁻¹ x min⁻¹; in Sf9-ABCC4 membrane vesicles, without GSH: 1; with GSH 2.5 pmol x mg protein⁻¹ x min⁻¹.
GSH increased ATP-dependent transport of LTB₄ into membrane vesicles from V79, HEK293, and Sf9 cells containing recombinant ABCC4. ATP-dependent transport of [³H]LTB₄ at a concentration of 100 nM was measured in membrane vesicles from the ABCC4- and vector-transfected V79 cells (Fig. 2A and B). The rates of ABCC4-mediated [³H]LTB₄ transport increased 4.7-fold in the presence of 5 mM GSH (Fig. 2B). A low rate of LTB₄ transport was detected in membrane vesicles from vector-transfected V79 cells (Fig. 2B), which is in line with the presence of hamster Abcc4 in the V79 cells (Rius et al., 2003; Rius et al., 2006). Similar results were obtained with the non-reducing GSH analog S-methyl GSH (data not shown). The LTB₄ transport in the presence of GSH was not significantly affected by DTT when present at a concentration of 1 mM.

Using membrane vesicles from the ABCC4- and vector-transfected HEK293 cells, the effects of GSH on the ATP-dependent transport of [³H]LTB₄ were similar to those with the V79 cells (Fig. 2C and D). In the absence of GSH, LTB₄ transport was negligible (Fig. 2C); however, addition of GSH caused an increase of the LTB₄ transport into membrane vesicles from both HEK-ABCC4 and HEK-Co cells (Fig. 2D). The transport rates in the presence of GSH were proportional to the corresponding levels of recombinant and endogenous ABCC4 protein in HEK293 cells (Fig. 1C). Membrane vesicles from Sf9 cells expressing recombinant ABCC4 confirmed the ABCC4-mediated LTB₄ transport in the presence of GSH (Fig. 3A).

Thus, with three different cellular sources of recombinant ABCC4, ABCC4 was recognized as an ATP-dependent transporter of LTB₄ in the presence but not in the absence of GSH (Fig. 2 and Fig. 3A). Similarly as shown earlier for the ABCC4-mediated cotransport of C-tau and GSH (Rius et al., 2003; Rius et al., 2006), GSH could be replaced in the LTB₄ transport assays by S-methyl GSH (not shown).

GSH increased ATP-dependent transport of LTB₄ into membrane vesicles from human platelets, U937, and HeLa control cells containing endogenous ABCC4. The
ABCC4-mediated transport of [\(^3\)H]LTB\(_4\) was further examined by transport studies with membrane preparations containing endogenous ABCC4. Using vesicles from plasma membranes of human platelets, we observed a significant ATP-dependent transport of [\(^3\)H]LTB\(_4\) only when GSH was present (Fig. 3C). To verify whether the low level of endogenous ABCC1 and ABCC5 could play a role in the LTB\(_4\) transport in membrane vesicles from platelets, [\(^3\)H]LTB\(_4\) transport was measured in vesicles from ABCC5-containing V79 cells and from ABCC1-containing HeLa cells (Leier et al., 1994a). Neither ABCC5 nor ABCC1 mediated a significant [\(^3\)H]LTB\(_4\) accumulation in vesicles in the presence or absence of GSH when compared to control vesicles. Moreover, we did not detect in human platelets by immunoblot analysis significant amounts of other ABCC subfamily members, including ABCC2, ABCC3, ABCC6, and ABCC11 (data not shown).

However, vesicles from U937, a myelomonocytic cell line, and HeLa control cells showed a pronounced and time-dependent ATP-dependent LTB\(_4\) transport in the presence of GSH (Fig. 3B and D). These results indicate that LTB\(_4\) transport in the presence of GSH by vesicles from human platelets, U937, and HeLa cells is mediated by endogenous ABCC4.

**ATP-dependent transport of LTC\(_4\) and LTD\(_4\) into membrane vesicles from V79, HEK293, and Sf9 cells, and from human platelets, U937, and HeLa cells.** To further characterize the substrate specificity of ABCC4 for leukotrienes, we measured transport of [\(^3\)H]LTC\(_4\) in vesicles containing recombinant ABCC4 from V79 and Sf9 cells during a 5-min period (Fig. 4A and B). Surprisingly, ATP-dependent LTC\(_4\) accumulation at a concentration of 100 nM amounted to 32.3 ± 3.2 pmol x mg protein\(^{-1}\) after 5 min in vesicles from ABCC4-transfected V79 cells and to 14 ± 0.5 pmol x mg protein\(^{-1}\) in vesicles from vector-transfected V79 cells (Fig. 4A). The accumulation of LTC\(_4\) resulted in 2.2-fold higher transport rates in vesicles from V79-ABCC4 cells than in vesicles from V79-Co cells. This ABCC4-mediated transport was confirmed in vesicles containing recombinant ABCC4 from Sf9 cells (Fig. 4B).
and from HEK293 cells. To further characterize the LTC₄ transport in membranes containing endogenous ABCC4, vesicles from human platelets were used (Fig. 4C). These vesicles, which contained negligible levels of ABCC1 (Fig. 1A and B), mediated a significant transport of LTC₄ with a transport rate of 0.6 pmol x mg protein⁻¹ x min⁻¹ (Fig. 4C). Membrane vesicles from U937 and HeLa cells, which also showed very low levels of endogenous ABCC1 (Fig. 1A), supported LTC₄ transport at a high rate (Fig. 4D). Addition of GSH or S-methyl GSH did not further stimulate the transport of LTC₄ in vesicles containing recombinant or endogenous ABCC4 (data not shown).

ATP-dependent transport of [³H]LTD₄, which is structurally very close to LTC₄, was also observed in vesicles from V79-ABCC4 cells with a rate of 1.0 pmol x mg protein⁻¹ x min⁻¹ in the absence of GSH (Fig. 5A). Interestingly, the ABCC4-mediated transport of LTD₄ was stimulated by GSH with a rate of 3.1 pmol x mg protein⁻¹ x min⁻¹ in vesicles from V79-ABCC4 (Fig. 5B). Membrane vesicles from platelets and U937 cells showed also LTD₄ transport with rates of 0.1 and 1.6 pmol x mg protein⁻¹ x min⁻¹, respectively, and the addition of GSH also stimulated LTD₄ transport with rates of 0.2 and 2.5 pmol x mg protein⁻¹ x min⁻¹, respectively.

Kinetic analysis of ABCC4-mediated ATP-dependent LTB₄ transport in the presence of GSH and of ATP-dependent LTC₄ transport. To determine the Kₘ value of ABCC4 for LTB₄, we used vesicles containing recombinant ABCC4 from V79 cells and vesicles containing endogenous ABCC4 from human platelets (Table 1). We tested the Kₘ values using a concentration range from 0.1 to 1 µM LTB₄ in the presence of 5 mM GSH (Table 1). The Vₘₐₓ values were 9.7 and 1.5 pmol x mg protein⁻¹ x min⁻¹ in vesicles from V79-ABCC4 and from platelets, respectively. The effect of GSH concentrations between 1 and 10 mM on the ATP-dependent [³H]LTB₄ transport was studied in vesicles from V79-
ABCC4 cells. At a constant concentration of 100 nM LTB₄, the $K_m$ value of GSH for ABCC4 was $1.1 \pm 0.2$ mM.

The $K_m$ value of ABCC4 for LTC₄ was also determined in vesicles containing recombinant ABCC4 from V79 cells (Table 1) and vesicles containing endogenous ABCC4 from platelets (Table 1). The $K_m$ value for LTC₄ in vesicles from V79 control cells was $0.13 \pm 0.01$ µM. Because of the similarity of the $K_m$ values in vesicles from V79-Co and V79-ABCC4, we did not correct the values for V79-ABCC4 vesicle-mediated transport by the data obtained by the control vesicles. The $V_{\text{max}}$ values were 37.4 pmol x mg protein⁻¹ x min⁻¹ for V79-ABCC4 and 9.7 pmol x mg protein⁻¹ x min⁻¹ for human platelets.

**Inhibition of the ABCC4-mediated LTB₄ transport in the presence of GSH in membrane vesicles from V79-ABCC4 cells and of the ABCC4-mediated LTC₄ transport in membrane vesicles from human platelets.** Several organic anions were tested for inhibition of the ATP-dependent [³H]LTB₄ transport in the presence of GSH into vesicles from V79-ABCC4 cells (Table 2). At a concentration of 1 µM, 5- and 12-hydroxyeicosatetraenoate did not cause a significant inhibition in the presence of GSH. In contrast, 1 µM LTC₄ was a potent inhibitor of the transport, causing 61 % inhibition, 1 µM LTD₄ caused 33 % inhibition, and the GSH derivative and LTC₄ analog, S-decyl glutathione at 10 µM caused 74 % inhibition. The non-steroidal anti-inflammatory drugs sulindac sulfide and indomethacin caused 74 % and 68 % inhibition, respectively, at 10 µM (Table 2). In the presence of GSH, the LTD₄ receptor antagonists MK571 and montelukast were also effective inhibitors of the LTB₄ transport. MK571 at 20 µM caused an inhibition of LTB₄ transport in the presence of GSH with a $K_i$ value of 9.8 µM. In addition, several known ABCC4 substrates and ABCC inhibitors produced a significant inhibition (Table 2).

In vesicles from human platelets, LTC₄ transport was measured in the presence of several organic anions, especially LTD₄ receptor antagonists (Table 3). Montelukast and
MK571 at a concentration of 20 µM caused 43 and 32% inhibition, respectively. In addition, S-decyl glutathione caused 58% inhibition at a concentration of 1 µM. Sulindac sulfide, in the absence of GSH and at a concentration of 10 µM, did not inhibit the LTC₄ transport in platelets. However, sulindac sulfide caused 90% inhibition in the presence of 5 mM GSH (Table 3). Additional compounds were identified to show a GSH-dependent inhibition. Accordingly, LTB₄, LTD₄, and C-tau caused inhibition only in the presence of GSH (Table 3). The presence of 5 mM GSH did not inhibit LTC₄ transport in the absence of inhibitors and the transport rates of LTC₄ were similar in the presence and absence of GSH.
Discussion

LTB₄ is an important mediator of the inflammatory process. In this study, we have identified ABCC4 as a novel and so far as the only efflux pump for LTB₄. Our conclusions are based on experiments in several membrane vesicle preparations that contain recombinant or endogenous human ABCC4 (Figs. 1–5). Moreover, millimolar concentrations of GSH were an absolute requirement for the ATP-dependent transport of LTB₄ (Figs. 2 and 3), analogous to the ATP-dependent transport of most bile acids by ABCC4 (Rius et al., 2003; Rius et al., 2006). In this study, we did not determine whether GSH is co-effluxed with LTB₄, as is the case with cholytaurine (Rius et al., 2003; Rius et al., 2006) or whether binding of GSH or S-methyl GSH to ABCC4 is sufficient. The LTB₄ efflux via ABCC4 in the presence of GSH is physiologically relevant, since millimolar concentrations (1 to 10 mM) of GSH occur in living cells (Meister, 1988), including PMNs (Oliver et al., 1976), erythrocytes (Kondo et al., 1980), and platelets (Maclouf and Murphy, 1988).

Our finding that LTC₄ is a full substrate for ABCC4 in the absence of GSH (Fig. 4, Table 1) is consistent with the structural differences between the molecules: LTB₄ lacks the covalently bound GSH moiety of LTC₄. The low Kᵢ value of the LTC₄ transport by ABCC4 (Fig. 4 and Table 1) contributes to an explanation of the active efflux transport of LTC₄ observed with blood platelets (Sjölinder et al., 1999) and erythrocyte membrane vesicles (Pulaski et al., 1996). Both platelets and erythrocytes contain much ABCC4 and hardly any ABCC1 (Fig. 1A and B). The abundance of ABCC4 observed in the plasma membrane of these cells confirms recent studies from other laboratories (Klokouzas et al., 2003; Jedlitschky et al., 2004). Our results suggest that all ATP-dependent LTC₄ efflux from platelets and erythrocytes is mediated by ABCC4. The high transport efficiency of ABCC4 in ATP-dependent LTC₄ export is indicated by the relatively high Vₘₕ/Kᵢ ratio amounting to 30 in platelet vesicles and 288 in vesicles from V79-ABCC4 fibroblasts (Table 1).
Efflux of LTC₄ from platelets and of LTB₄ from erythrocytes and endothelial cells represents the final step in the transcellular synthesis of these LTs from their precursor LTA₄ (Maclouf and Murphy, 1988; Sjölinder et al., 1999; Folco and Murphy, 2006). ABCC4 may thus play an important role in the transcellular synthesis of LTB₄ and LTC₄. ABCC4 may be termed an eicosanoid efflux pump in view of its broad substrate spectrum that includes, in addition to LTB₄ and LTC₄, prostaglandin (PG) E₂ (Reid et al., 2003), PGF₂α, and thromboxane B₂ (Rius et al., 2005).

In addition, ABCC4 expression was detected in human PMNs and in the human myelomonocytic leukaemia cell line U937 (Fig. 1D). Confocal immunofluorescence microscopy indicated localization of ABCC4 in both cell lines in the plasma membrane and in intracellular vesicles. Further studies are required to determine whether the stimulation of PMNs and U937 with the calcium ionophore A23187 enhances the sorting of ABCC4 to the plasma membrane. The observation that membrane vesicles from U937 show ATP-dependent transport of LTB₄ in the presence of GSH (Fig. 3D), of LTC₄ (Fig. 4D), and of LTD₄ indicates that ABCC4 is at least partially localized to the plasma membrane and to plasma membrane derived vesicles. In this study we have not attempted to isolate inside-out membrane vesicles from human PMNs for additional experiments on LTB₄ transport.

Our work adds several inhibitors of ABCC4-mediated transport to those identified in recent years (van Aubel et al., 2002; Rius et al., 2003; Reid et al., 2003; Jedlitschky et al., 2004). In this study, potent inhibitory compounds of ATP-dependent transport of LTB₄ in the presence of 5 mM GSH included LTC₄ and its structural analog S-decyl GSH, the anti-inflammatory agent sulindac sulfide, and the LTD₄ receptor antagonists montelukast and MK571 (Table 2). The latter substances also interfered with the ABCC4-mediated transport of LTC₄ in the absence of GSH (Table 3). Some of these compounds were identified formerly as inhibitors of ABC1, as described for MK571 (Jedlitschky et al., 1994; Leier et al., 1994a; Keppler et al., 1998) and S-decyl GSH (Loe et al., 1996). Thus, the inhibitory selectivity of
these structural analogs of LTD₄ and LTC₄ may not be sufficient to discriminate between ABCC1 and ABCC4. This observation is surprising since both ABC transporters share only 39% amino acid sequence identity and differ largely by the absence of the amino-terminal extension in ABCC4 (Deeley et al., 2006). Several of the inhibitors of LTB₄ transport by ABCC4 (Table 2) are established anti-inflammatory agents, such as indomethacin and sulindac sulfide. Inhibition of ABCC4 and thereby interference of the transcellular synthesis of LTB₄ and LTC₄ may contribute to the anti-inflammatory action of some anti-inflammatory drugs. However, anti-inflammatory agents must first be taken up by a target cell before they can interact with ABCC4 on its cytosolic domain, which was exposed in our inhibition studies with inside-out membrane vesicles. Moreover, the distribution and substrate specificity of the cellular uptake transporters and the site of drug administration in the body may determine whether an inhibitor of ABCC4 exerts anti-inflammatory actions.

Over the past years, ABCC4 has been recognized as a transporter of broad substrate specificity, including endogenous substrates, drugs, and drug candidates (Lee et al., 2000; van Aubel et al., 2002; Rius et al., 2003; Reid et al., 2003; Jedlitschky et al., 2004; Rius et al., 2005; Rius et al., 2006). The physiological function of human ABCC4, however, cannot be defined by the in vitro substrate specificity alone. Rather, that role may depend on the cell type and the level of ABCC4 expression, in addition to the presence or absence of other ABCC subfamily members, such as ABCC1, ABCC2, or ABCC3. Unfortunately, information from Abcc4-knock-out mice cannot serve to define the physiological role of this transporter in humans because of the large kinetic differences between human and murine ABCC4/Abcc4 (Wolf de et al., 2007). Our present work was focused on three novel substrates of ABCC4 and indicated that this eicosanoid transporter may play an important role in the biosynthetic release in human PMNs and in the transcellular biosynthesis of LTB₄ and LTC₄ in human platelets and erythrocytes. Studies on more potent and selective inhibitors of ABCC4 may be an attractive aim in drug development.
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References


Footnotes

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Legends for Figures

Fig. 1. Immunodetection of ABCC4 and ABCC1. A, immunoblot analysis of ABCC4 and ABCC1 in membrane vesicles from HL60-ADR, HeLa control (HeLa-Co), and U937 cells, in the plasma membrane fraction from human platelets, in membrane vesicles from human erythrocytes, and tissue homogenate from human prostate (10 µg protein per lane). The blots were immunostained using the polyclonal SNG antiserum against human ABCC4 (Rius et al., 2003), the monoclonal M4I-10 antibody against ABCC4, and the monoclonal QCRL-1 antibody against human ABCC1. Human prostate and HL60-ADR were used as positive controls for ABCC4 (Lee et al., 2000) and ABCC1, respectively. B, the plasma membrane fraction from human platelets was loaded in a range from 20 µg to 50 µg protein. Increasing amounts of ABCC4 protein were detected by the SNG antiserum and the M4I-10 antibody but ABCC1 signal intensity remained below detectability using the QCRL-1 antibody and equal exposure times for both blots. C, immunoblot analysis of ABCC4 using the SNG antiserum in membrane vesicles (30 µg per lane) from control cells (V79-Co, HEK-Co, and Sf9-Co) and from cells expressing recombinant ABCC4 (V79-ABCC4, HEK-ABCC4, and Sf9-ABCC4). D, confocal laser scanning micrographs of isolated human PMNs and U937 cells using the affinity-purified SNG antibody (green fluorescence) and propidium iodide to stain nuclei (red fluorescence). Upper right panel shows a magnified view of the area indicated in upper left panel. ABCC4 was mainly found in intracellular membranous structures but was also partially detected at the plasma membrane. Pre-incubation of the affinity-purified SNG antibody with the ABCC4 peptide abolished ABCC4 staining (lower left panel). Bars, 10 µm.

Fig. 2. GSH increases transport of LTB4 into membrane vesicles from ABCC4-transfected (V79-ABCC4 and HEK-ABCC4) and vector-transfected control (V79-Co and HEK-Co) cells. Membrane vesicles (30 µg of protein) were incubated with 100 nM [3H]LTB4 in the absence (A and C) or presence (B and D) of 5 mM GSH, and the vesicle-associated
radioactivity was determined by centrifugation through Sephadex G-50 columns. The rates of net ATP-dependent transport were calculated by subtracting transport in the presence of 4 mM AMP-PCP as a blank from transport in the presence of 4 mM ATP. Data represent mean values ± S.D. from a triplicate determination reproduced independently at least once. *P< 0.01 and **P<0.001 as compared to vesicles from vector-transfected control cells.

**Fig. 3.** Effect of GSH on transport of LTB₄ into membrane vesicles from Sf9 cells containing recombinant ABCC4 protein, from HeLa control cells, from human platelets, and from U937 cells. A, membrane vesicles (50 µg of protein) from Sf9 cells expressing recombinant ABCC4 (Sf9-ABCC4) and control Sf9 cells (Sf9-Co) were incubated with 100 nM [³H]LTB₄ in the presence of 5 mM GSH. B, membrane vesicles (30 µg of protein) from vector-transfected HeLa control (HeLa-Co) cells were incubated with 100 nM [³H]LTB₄ in the absence or presence of 5 mM GSH. C, membrane vesicles (100 µg of protein) from human platelets were incubated with 100 nM [³H]LTB₄ in the absence or presence of 5 mM GSH. D, membrane vesicles (30 µg of protein) from parental U937 cells were incubated with 100 nM [³H]LTB₄ in the absence or presence of 5 mM GSH. Radioactivity and ATP-dependent transport were determined as described in the legend to Fig. 2. Data represent mean values ± S.D. from a triplicate determination reproduced independently at least once. *P< 0.01 as compared with vesicles from control cells (A) and **P<0.001 as compared with the absence of GSH (B, C, and D).

**Fig. 4.** Transport of LTC₄ into membrane vesicles from V79 and Sf9 cells containing recombinant ABCC4 protein (V79-ABCC4 and Sf9-ABCC4), from human platelets and from HeLa control and U937 cells. A and B, membrane vesicles (30–50 µg of protein) from ABCC4-expressing cells (V79-ABCC4 and Sf9-ABCC4) and control cells (V79-Co and Sf9-Co) were incubated with 100 nM [³H]LTC₄. C and D, membrane vesicles from human
platelets (100 µg of protein), HeLa control (HeLa-Co) and U937 cells (25 µg of protein) were incubated with 100 nM [3H]LTC₄. The vesicle-associated radioactivity was determined by filtration through nitrocellulose filters. The rates of ATP-dependent transport were calculated by subtracting transport in the presence of 4 mM 5′-AMP as a blank from transport in the presence of 4 mM ATP. Data represent mean values ± S.D. from a triplicate determination reproduced independently at least once. *P<0.001 as compared with vesicles from control cells (A and B) or to the absence of ATP (C and D).

**Fig. 5.** Transport of LTD₄ into membrane vesicles from V79 cells containing recombinant ABCC4 protein (V79-ABCC4). Membrane vesicles (30 µg of protein) from ABCC4-expressing V79 cells (V79-ABCC4) and control cells (V79-Co) were incubated with 100 nM [3H]LTD₄ in the absence (A) or presence (B) of 5 mM GSH. Radioactivity and ATP-dependent transport were determined as described in the legend to Fig. 4. Data represent mean values ± S.D. from a triplicate determination reproduced independently at least once. *P<0.001 as compared with vesicles from control cells
Tables

TABLE 1

Rates of ATP-dependent transport of $[^3]H$LTB$_4$ in the presence of 5 mM GSH and of $[^3]H$LTC$_4$ were determined in membrane vesicles from $ABCC4$-transfected V79 cells and in plasma membrane fractions from human platelets under substrate concentrations described in “Materials and Methods”. The $K_m$ values were calculated from double-reciprocal plots. Data represent mean values ± S.D. from a triplicate determination.

<table>
<thead>
<tr>
<th>Leukotriene</th>
<th>$K_m$ (µM)</th>
<th>$V_{max}/K_m$ (µl x min$^{-1}$ x mg protein$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vesicles from V79-ABCC4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LTB$_4$ + 5 mM GSH</td>
<td>5.2 ± 0.2</td>
<td>1.9</td>
</tr>
<tr>
<td>LTC$_4$</td>
<td>0.13 ± 0.02</td>
<td>287.7</td>
</tr>
<tr>
<td>Vesicles from platelets</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LTB$_4$ + 5 mM GSH</td>
<td>5.6 ± 0.6</td>
<td>0.3</td>
</tr>
<tr>
<td>LTC$_4$</td>
<td>0.32 ± 0.03</td>
<td>30.3</td>
</tr>
</tbody>
</table>
Inhibition of ABCC4-mediated LTB₄ transport in the presence of GSH

Membrane vesicles from V79-ABCC4 cells were incubated with 100 nM [³H]LTB₄ and 5 mM GSH for 5 min at 37 °C with several compounds. Rates of ATP-dependent [³H]LTB₄ transport were determined as described and calculated as % of control. The rate of [³H]LTB₄ transport in the absence of inhibitors amounted to 0.75 ± 0.06 pmol x mg protein⁻¹ in 5 min. Data represent mean values ± SD from a triplicate determination.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Concentration (µM)</th>
<th>ATP-dep. [³H]LTB₄ Transport (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[³H]LTB₄ (control, 100 nM)</td>
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<td>100</td>
</tr>
<tr>
<td>5-Hydroxy-6,8,11,14-eicosatetraenoate</td>
<td>1</td>
<td>86 ± 7</td>
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<tr>
<td>12-Hydroxy-5,8,10,14-eicosatetraenoate</td>
<td>1</td>
<td>106 ± 6</td>
</tr>
<tr>
<td>LTC₄</td>
<td>1</td>
<td>39 ± 4</td>
</tr>
<tr>
<td>LTD₄</td>
<td>1</td>
<td>67 ± 9</td>
</tr>
<tr>
<td>S-Decyl GSH</td>
<td>10</td>
<td>26 ± 3</td>
</tr>
<tr>
<td>Sulindac sulfide</td>
<td>10</td>
<td>26 ± 3</td>
</tr>
<tr>
<td>Indomethacin</td>
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<td>32 ± 2</td>
</tr>
<tr>
<td>Prostaglandin E₂</td>
<td>10</td>
<td>46 ± 5</td>
</tr>
<tr>
<td>Cholyltaurine</td>
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<td>39 ± 2</td>
</tr>
<tr>
<td>Dipyridamole</td>
<td>10</td>
<td>57 ± 3</td>
</tr>
<tr>
<td>MK571</td>
<td>10</td>
<td>60 ± 4</td>
</tr>
<tr>
<td>Montelukast</td>
<td>10</td>
<td>52 ± 5</td>
</tr>
<tr>
<td>Probenecid</td>
<td>100</td>
<td>73 ± 7</td>
</tr>
<tr>
<td>Sulfinpyrazone</td>
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<td>73 ± 7</td>
</tr>
</tbody>
</table>
TABLE 3

Inhibition of ABCC4-mediated LTC₄ transport in platelets

Plasma membrane vesicles from human platelets were incubated with 100 nM [³H]LTC₄ for 3 min at 37 °C in the presence of several compounds at the concentrations indicated. Except where indicated with sulindac sulfide, [³H]LTC₄ transport was measured in the absence of GSH. Rates of ATP-dependent [³H]LTC₄ transport were determined as described and calculated as % of control. The rate of [³H]LTC₄ transport in the absence of inhibitors amounted to 2.8 ± 0.2 pmol x mg protein⁻¹ in 3 min. GSH itself at a concentration of 5 mM did not cause inhibition of LTC₄ transport. Data represent mean values ± SD from a triplicate determination.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Concentration</th>
<th>ATP-dep. [³H]LTC₄ Transport (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[³H]LTC₄ (control, 100 nM)</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>S-Decyl GSH</td>
<td>1</td>
<td>42 ± 2</td>
</tr>
<tr>
<td>LTB₄</td>
<td>5</td>
<td>126 ± 8</td>
</tr>
<tr>
<td>LTB₄ + 5 mM GSH</td>
<td>5</td>
<td>58 ± 7</td>
</tr>
<tr>
<td>LTD₄</td>
<td>5</td>
<td>96 ± 9</td>
</tr>
<tr>
<td>LTD₄ + 5 mM GSH</td>
<td>5</td>
<td>54 ± 7</td>
</tr>
<tr>
<td>Cholyltaurine</td>
<td>10</td>
<td>97 ± 7</td>
</tr>
<tr>
<td>Cholyltaurine + 5 mM GSH</td>
<td>10</td>
<td>55 ± 7</td>
</tr>
<tr>
<td>Sulindac sulfide</td>
<td>10</td>
<td>108 ± 2</td>
</tr>
<tr>
<td>Sulindac sulfide + 5 mM GSH</td>
<td>10</td>
<td>2 ± 0.5</td>
</tr>
<tr>
<td>MK571</td>
<td>20</td>
<td>68 ± 3</td>
</tr>
<tr>
<td>Montelukast</td>
<td>20</td>
<td>57 ± 4</td>
</tr>
</tbody>
</table>
Figure 2

- GSH

V79-ABCC4/Co
100 nM LTB₄ without GSH

V79-ABCC4

V79-Co

+ GSH

V79-ABCC4/Co
100 nM LTB₄ + 5 mM GSH

V79-ABCC4

V79-Co

HEK-ABCC4/Co
100 nM LTB₄ without GSH

HEK-ABCC4

HEK-Co

HEK-ABCC4

HEK-Co

HEK-ABCC4

HEK-Co

ATP-dep. [³H]LTB₄ Transport (pmol/mg protein)

Times (min)
Figure 5

A) V79-ABCC4/Co
100 nM LTD₄
without GSH

B) V79-ABCC4/Co
100 nM LTD₄
+ 5 mM GSH

ATP-dep. [³H]LTD₄ Transport (pmol/mg protein)

Times (min)