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Formation and Efflux of ATP-Binding Cassette Transporter Substrate 2,4-Dinitrophenyl-*S*-Glutathione from Cultured Human Term Placental Villous Tissue Fragments

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Abstract: Upon exposure to 1-chloro-2,4-dinitrobenzene (CDNB), the human placental tissue forms its glutathione conjugate 2,4-dinitrophenyl-*S*-glutathione (DNP-SG). The purpose of this study was to investigate the involvement of human placental ATP-binding cassette (ABC) transporters in the efflux of DNP-SG. Placental tissue samples were obtained from pregnant patients undergoing C-section deliveries following normal pregnancies; villous tissue was cultured in suspension, and DNP-SG formation and efflux upon exposure to 100 μM CDNB were measured by HPLC. DNP-SG efflux decreased by 69.1 ($\pm$11.3)%, 51.1 ($\pm$5.4)%, 56.7 ($\pm$8.3)% and 53.6 ($\pm$10.8)% ($p < 0.05$) in the presence of 5 mM sodium orthovanadate (ATPase inhibitor), 100 μM MK571 (MRP-inhibitor), 1 mM dipyridamole (BCRP/P-gp/MRP1-inhibitor) and 100 μM verapamil (P-gp/MRP1 inhibitor) respectively, without any change in DNP-SG formation, total tissue glutathione, GSH/GSSG ratio, tissue integrity or tissue viability. These data clearly established the role of ABC transporters in the human placental efflux of DNP-SG. To investigate the contribution of various ABC transporters toward DNP-SG transport, ATP-dependent transport of ^{3}H-DNP-SG was determined in *Sf*9 membrane vesicles overexpressing P-gp, BCRP and the MRP proteins. MRP1-mediated DNP-SG transport was inhibited in the presence of sodium orthovanadate, MK571, dipyridamole and verapamil in the presence of glutathione. Furthermore, MRP1-mediated transport [$K_t = 11.3 \pm 1.3$ μM and $v_{max} = 86.7 \pm 1.9$ pmol/mg/min] was a high-affinity process compared to MRP2-mediated transport [$K_t = 168 \pm 7$ μM and $v_{max} = 1367 \pm 18$ pmol/mg/min]. The inhibition pattern and the kinetics of DNP-SG efflux in the placental villous tissue were consistent with MRP1-mediated DNP-SG efflux, suggesting a functional role and an apical localization for an MRP1-like transporter in the human placental syncytiotrophoblast.

Keywords: 1-Chloro-2,4-dinitrobenzene; CDNB; 2,4-dinitrophenyl-*S*-glutathione; DNP-SG; ATP-binding cassette transporters; GSTP1-1; MRP1; MRP2; placental syncytiotrophoblast

1. Introduction

The placenta serves as the link between the mother and the fetus and acts as a multifunctional organ responsible for supply of nutrients and transfer of waste products, limits the transfer of some xenobiotics to the fetus and secretes physiological compounds such as hormones and prostaglandins. The human placenta contains a multinucleated syncytiotrophoblast layer that separates the fetal circulation from the maternal circulation, as seen in the cross section of the chorionic villum in Figure 1. The apical (brush border or maternal) surface of the syncytiotrophoblast is bathed in maternal blood. The basolateral (or fetal) surface is in contact with the discontinuous cytotrophoblast or the fetal connective tissue. Fetal capillaries that are relatively leaky are embedded in the fetal connective tissue and are present in the center.

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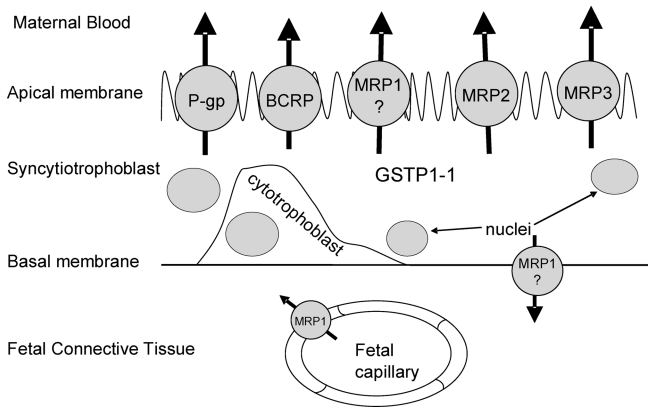

Figure 1. Cross sectional schematic view of the human placental chorionic villous tissue. (Adapted from Leslie et al.[1])

Thus, transfer of nutrients or xenobiotics across the placenta from the mother to the fetus would require translocation across the apical and basolateral membranes of the syncytiotrophoblast and across the fetal capillary endothelium.[1−3]

ATP-binding cassette (ABC) transporters such as P-glycoprotein (P-gp), breast cancer resistance protein (BCRP) and multidrug resistance associated proteins (MRP 1, 2 and 3) have been detected on the apical side of the placental syncytiotrophoblast and/or the fetal capillary endothelium (Figure 1). These transporters may play an important role in protecting the fetus from xenobiotic exposure, and secreting physiologic waste products such as bilirubin glucuronides from the fetal circulation toward the maternal circulation.[2,4] While the protective role of P-gp in the human placenta has been clearly established,[5−11] there is lesser evidence of the functional activity of BCRP[12−14] and the MRPs[2,15] in the human placenta.

The objective of this study was to investigate the involvement of BCRP or the MRPs in placental xenobiotic defense mechanisms. To address this question, we investigated whether BCRP and/or any of the MRPs and GSTP1-1 can operate in a coordinated fashion to detoxify 1-chloro-2,4-dinitrobenzene (CDNB) from cultured normal human term placental villous tissue fragments. The human placental GSTP1-1 conjugates CDNB, which is a toxic xenobiotic known to cause oxidative stress and cell death, with glutathione, to form 2,4-dinitrophenyl-*S*-glutathione (DNP-SG), which is a well-established probe substrate for MRPs 1, 2 and 3.[16−19] MRP mediated efflux of DNP-SG is extremely efficient and has been well characterized in several

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in vitro models as well as animal tissues.[20−26] There is limited evidence supporting DNP-SG as a substrate for BCRP,[27] but BCRP is highly expressed in the human placenta and hence may play a role in DNP-SG efflux from the placenta. The activity of GSTP1-1 toward CDNB metabolism in the human placenta has been studied;[28] however the mechanism of efflux of DNP-SG from the placental tissue is not clearly established. Thus, this model would enable the study of the coordinated metabolism and efflux functions of the placenta.

Previously, we reported the formation and efflux of DNP-SG in the human placental villous tissue, without any significant formation of further downstream metabolites such as 2,4-dinitrophenyl-*S*-cysteinylglycine, 2,4-dinitropheyl-*S*-cysteine and 2,4-dinitrophenyl-*N*-acetylcysteine after brief exposure to CDNB. DNP-SG efflux increased linearly with time up to 45 min and was inhibited in the presence of ATPase inhibitor sodium orthovanadate. These data suggested a coordinated role of GSTP1-1 and ABC transporters in CDNB disposition in the placenta.[29] Recently, we validated the placental villous tissue culture model by studying the effect of time in culture on tissue morphology, viability, integrity, total tissue glutathione content and protein expression of P-gp, MRP2, BCRP and GSTP1-1. Protein expression of BCRP, GSTP1-1, villous tissue structure and MTT incorporation remained unchanged over 48 h in culture. Expression of P-gp, MRP2 and total tissue glutathione decreased with time in culture. LDH release was unchanged up to 24 h and increased at 48 h suggesting a decline in tissue integrity at 48 h. However, DNP-SG buffer/total ratio, DNP-SG formation and the extent of inhibition of DNP-SG efflux by sodium orthovanadate showed only a few minor changes through 48 h.[30]

DNP-SG serves as a probe representing other relevant glutathione conjugates of endobiotics such as leukotriene C4, 4-hydroxynonenal-*S*-glutathione, as well as glutathione conjugates of xenobiotics including 3,4-methylenedioxymethamphetamine (“ecstasy”), and dietary polyphenols. The purpose of the current study was to investigate the relative contribution of various apical ABC transporters toward DNP-SG efflux from the cultured human term placental villous tissue. We studied the effect of ABC transporter inhibitors, sodium orthovanadate (ATPase inhibitor), MK571 (MRP-inhibitor),[31] dipyridamole (DP; BCRP/P-gp/MRP1-inhibitor)[32,33] and verapamil (P-gp inhibitor, MRP1 inhibitor in the presence of glutathione),[34] on DNP-SG formation and efflux in the villous tissue, and on MRP1/2-mediated ATP-dependent ^{3}H-DNP-SG transport in inside-out *Sf*9 membrane vesicles. The kinetics and the observed inhibition pattern for ^{3}H-DNP-SG efflux from *Sf*9 membrane vesicle studies supported the findings from the villous tissue culture model and are consistent with MRP1-mediated efflux, suggesting a functional role and an apical localization for an MRP1-like transporter in the placental syncytiotrophoblast.

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2. Experimental Section

2.1. Materials. CDNB (99% purity), 2-vinylpyridine (>97% purity) and perchloric acid (PCA) reagent (70% in water) were purchased from Acros Organics (Morris Plains, NJ). 1-Fluoro-2,4-dinitrobenzene; FDNB (99% purity) was obtained from Fluka Biochemika (Buchs, Switzerland). Ethacrynic acid (EA) and dipyridamole (DP) were purchased from MP Biomedicals, LLC (Solon, OH). MK571 was obtained from Alexis Biochemicals (San Diego, CA). Tritium labeled [glycine-2-^{3}H]-glutathione (38.6 Ci/mmol) and [^{3}H]-estradiol 17-(β-D-glucuronide) [^{3}H-$E_2$17G] (45.8 Ci/mmol) were obtained from Perkin-Elmer (Boston, MA). Both radiolabels were of purity >97%. ^{3}H-DNP-SG was synthesized as previously described by incubating [glycine-2-^{3}H]-glutathione (0.65 nmol) with FDNB (0.5 μmol).[22] The product was treated with 10% PCA in water and Dulbecco's phosphate buffered saline (DPBS) (1:1 v/v) and was centrifuged, and ^{3}H-DNP-SG in the supernatant was identified by HPLC as described earlier,[29] with reference to the unlabeled DNP-SG standard. Fractions corresponding to ^{3}H-DNP-SG were collected from the eluent, dried under nitrogen, resuspended in ethanol and stored at 4 °C. Purity of the synthesized ^{3}H-DNP-SG was tested by HPLC and was >95%. All other chemicals, solvents or reagents were of analytical grade and were obtained from Sigma-Aldrich (St. Louis, MO) or Fisher Chemicals (Fair Lawn, NJ) unless indicated below.

2.2. Human Subjects. The study was approved by the VCU Institutional Review Board, and informed consent was obtained from patients prior to delivery. Placental tissue samples were obtained from ten women (between ages of 18−45 years, gestational length ≥36 weeks) within 30 min of birth from cesarean section deliveries following normal pregnancies at the VCU Medical Center Hospital. Patients with diabetes, preeclampsia, hypertension, febrile illness, HIV infection, or history of smoking, drug or alcohol abuse were excluded. Patients' medical records were accessed to record the maternal age, parity, weight, race, obstetric course, medical history, neonatal birth weight, time of birth, Apgar scores at the time of birth, and gross placental abnormalities.

2.3. Placental Villous Tissue Fragments: Culture Conditions. Placental villous tissue was processed under sterile conditions on ice. Tissue weight was recorded, and tissues with gross abnormalities such as absent cotyledons, discolorations on the maternal or fetal surfaces were excluded from the study. Triangular wedges (approximately 100 g) starting from the point of cord insertion extending out to the periphery were cut, decidual layer and chorionic plates were removed, and the tissue was rinsed with ice-cold sterile saline. After complete removal of blood from intervillous spaces by several cycles of mincing and washing with DPBS with antibiotics (200 U/mL penicillin, 200 μg/mL streptomycin, 500 U/ml nystatin), tissue fragments (300−400 mg) were cultured up to 48 h in 7 mL M199 medium (Invitrogen, Carlsbad, CA) supplemented with 2 mM L-glutamine and 10% fetal bovine serum in 6-well polystyrene dishes at 37 °C, under 95% air/5% CO_2.

2.4. Effect of GSTP1-1 or ABC Transporter Inhibition on DNP-SG Formation or Efflux. To investigate the involvement of human placental ABC transporters in the efflux of DNP-SG, villous tissue (n = 6 patients) was cultured in M199 medium for 6−10 h, preincubated without or with ethacrynic acid (GST inhibitor; 0.15−1.5 mM),[35] sodium orthovanadate (ATPase inhibitor; 5 mM), MK571 (MRP inhibitor; 30−300 μM), dipyridamole (BCRP/P-gp/MRP1-inhibitor; 0.3−3 mM) or verapamil (P-gp inhibitor and MRP1 inhibitor in the presence of glutathione; 30−300 μM) for 1 h at 37 °C and then exposed to 100 μM CDNB for 5 min at 10 °C, rinsed twice with DPBS and incubated at 37 °C without or with respective pretreatment inhibitors. Buffer and tissue samples were collected in 10% PCA in water (1:1 v/v) at 20 min; DNP-SG and CDNB were quantified by a previously validated HPLC assay followed by UV detection.[29] The lower limit of quantitation (LLOQ) and linear range for the assay were 1 μM and 1−20 μM for CDNB, and 0.1 μM and 0.1−100 μM for DNP-SG, respectively, following a 100 μL injection for quantification from both human placental tissue homogenate and buffer.

To characterize the formation and efflux of DNP-SG, villous tissue (n = 3 patients) was cultured up to 48 h in M199 medium, preincubated for 1 h without or with sodium orthovanadate, exposed to 3−1000 μM CDNB for 5 min at 10 °C, rinsed twice in DPBS to remove extracellular CDNB and incubated in DPBS with shaking at 37 °C. CDNB and DNP-SG from buffer and tissue samples were assayed as previously described.[29]

The effect of sodium orthovanadate treatment on tissue viability was assessed by measuring the incorporation of thiazolyl blue tetrazolium bromide (MTT) for n = 3 patients. Furthermore, the effect of inhibitors on the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG); GSH/GSSG ratio (n = 3 patients) and lactate dehydrogenase (LDH) release (n = 1 patient) was assessed by collecting tissue and preincubation media samples after a 1 h incubation with individual inhibitors in culture medium at 37 °C.

2.5. Biochemical Assays. Expression of placental ABC transporters MRP1, MRP2, P-gp, BCRP, and enzyme GSTP1-1 as well as housekeeping genes actin and β-actin was assessed by immunoblotting as previously described.[36] Minced villous tissue samples (200−300 mg) were collected immediately after processing the tissue (prior to villous tissue culture) and frozen at −80 °C. At a later time, tissue samples were thawed on ice and homogenized in 1:10 w/v buffer (50 mM Tris, 1 mM EDTA, 1 mM PMSF, 5 μg/mL leupeptin, 5 μg/mL aprotinin, 2 μg/mL antipain, 2 μg/mL pepstatin A, 1 mg/mL soya trypsin inhibitor, pH 7.4 at 4 °C) using a Polytron PT 10-35 homogenizer with a PTA 10

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TS generator (Kinematica, Lucerne, Switzerland; speed setting 6.5), for 30 s on ice. Tissue homogenate samples were centrifuged at 6000*g* for 5 min at 4 °C, and 500 μL aliquots were snap frozen by dropping into liquid nitrogen and stored at −80 °C. Protein concentrations were determined by a modification of the Lowry protein assay using bovine serum albumin as a standard.[37] For immunoblotting, proteins were denatured in the presence of sodium dodecyl sulfate at 37 °C for 30 min before loading onto 4−20% Precise protein gels (Pierce, Rockford, IL), separated by standard electrophoresis in the presence or absence of NuPage sample reducing agent (Invitrogen) and transferred onto nitrocellulose membranes (Schleicher and Schuell, Keene, NH). *Sf*9 protein (0.05 μg) from in-house membrane vesicles individually overexpressing MRP2, P-gp or BCRP; or commercially purchased sucrose fractionated *Sf*9 membrane vesicles overexpressing MRP1 (BD Biosciences, San Jose, CA) were used as positive controls. Membranes were blocked overnight at 4 °C using Odyssey blocking buffer (Li-Cor Biosciences, Lincoln, NE). Binding of the primary antibodies [mouse anti-human MRP1 (MRPm6; 1:200), mouse anti-human MRP2 (M_2-III6; 1:4000), mouse anti-human BCRP (BXP-21; 1:1000) (Alexis Biochemicals, San Diego, CA), mouse anti-human P-gp (F4; 1:2000) (Kamiya Biomedical Co., Seattle, WA), mouse anti-β-actin (1:2000), rabbit anti-actin (1:1000) (Sigma), rabbit anti-GSTP1-1 pAb (1:2000) (Calbiochem, La Jolla, CA)] and the secondary antibodies [goat anti-mouse IR Dye 800, goat anti-rabbit Alexa Fluor 680 (Li-Cor)] was performed in the Odyssey blocking buffer at room temperature for 1 h, in the dark. The resultant fluorescent complexes were detected and the band intensity was quantified using the Odyssey Infrared Imaging System and the Odyssey Application Software, version 2.1 (Li-Cor).

Effect of sodium orthovanadate treatment on tissue viability was assessed by the MTT assay as described previously.[38] After 48 h in culture, tissue samples (200−300 mg, unfrozen) from $n = 3$ patients were collected in triplicate after a 1 h incubation without or with 5 mM sodium orthovanadate and exposed to MTT, and the formation of the formazan product of MTT was measured by monitoring relative absorbance at 595 nm using the Synergy 2 Multi-Detection Microplate Reader (BioTek Instruments, Inc., Winooski, VT).

LDH release into the tissue culture media was measured as a marker for tissue integrity after treatment with various inhibitors in a representative patient. After 6−10 h in culture, media samples (200 μL) were collected in triplicate after 1 h incubation with respective pretreatment inhibitors and frozen at −20 °C. At a later time, LDH release was measured using the LDH UV-Rate assay kit (Stanbio Laboratory, Boerne, TX).

GSH/GSSG ratio was determined ($n = 3$ patients) as described previously.[39,40] After 6−10 h in culture, tissue samples (100−200 mg) were collected in triplicate each in the absence (total glutathione) or presence (oxidized glutathione) of 100 μL of 2-vinylpyridene diluted in absolute ethanol (1:1 v/v), after a 1 h incubation with the respective pretreatment inhibitors (except dipyridamole), and frozen at −80 °C. At a later time, samples were homogenized as described earlier for immunoblotting. Tissue homogenates were treated with 10% sulfosalicylic acid (1:1 v/v) and incubated with Ellman's reagent in the presence of glutathione reductase enzyme and β-NADPH at room temperature. The reaction was monitored by measuring the absorbance at 405 nm using the Synergy 2 Multi-Detection Microplate Reader (BioTek). Reduced glutathione was used as a standard.

2.6. ^{3}H-DNP-SG or ^{3}H-$E_2$17G Transport into Inside-Out *Sf*9 Membrane Vesicles. *Sf*9 membrane vesicles overexpressing human isoforms of P-gp, BCRP and MRP2, as well as control membrane vesicles (EV; empty virus lacking the coding regions for the ABC transporters), were prepared in house as previously described,[36,41−43] and *Sf*9 membrane vesicles overexpressing MRP1 and MRP3 were purchased from BD Biosciences (San Jose, CA). Transport experiments were performed, in Tris-sucrose buffer, containing 5 mM ATP or AMP, 10 mM $MgCl_2$, 10 mM phosphocreatine, 100 μg/mL creatine phosphokinase in the presence or absence of unlabeled DNP-SG (0.3−1000 μM), sodium orthovanadate (0.5 mM), MK571 (100 μM), dipyridamole (100 μM), verapamil (100 μM) with or without glutathione (0.5 mM) in dimethyl sulfoxide (<0.75% v/v).[36,41,42] ATP-dependent transport of ^{3}H-DNP-SG (60 nM) or ^{3}H-$E_2$17G (49.6 nM) into membrane vesicles (5−20 μg/20 μL) was measured in incubations at 37 °C between 2 and 60 min. Transport was stopped by adding 3.5 mL of ice-cold stop buffer and filtering through Durapore 0.4 μm filters (Millipore Corporation, Bedford, MA). Filters were collected and mixed with scintillation cocktail (Ecoscint, National Diagnostics, Atlanta, GA), and ^{3}H was detected by liquid

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scintillation counting. Transport corrected for that in the presence of AMP was termed ATP-dependent transport.

2.7. Data Analysis. Data were plotted and analyzed using Prism version 5 (GraphPad Software Inc., San Diego, CA). Total DNP-SG content was determined by the summation of DNP-SG content in the efflux buffer and tissue DNP-SG, at the end of the experiment. Tissue CDNB content was estimated by the summation of the measured tissue CDNB content and the total DNP-SG content at the end of the experiment, assuming a 1:1 stoichiometric ratio for formation of DNP-SG from CDNB. Kinetics of DNP-SG formation were analyzed by unweighted nonlinear regression curve fitting to the Michaelis−Menten equation (Prism v5), where K_m represents the affinity for saturable DNP-SG formation in terms of tissue CDNB content normalized to tissue weight and υ_{max} represents the maximum rate for saturable DNP-SG formation. Kinetics of DNP-SG efflux were analyzed by unweighted nonlinear regression curve fitting to eq 1.

$$J = \frac{J_{max}S}{K_t + S} + k_d S \quad (1)$$

where J and S represent the DNP-SG transport rate and tissue DNP-SG content normalized to tissue weight, respectively. J_{max}, K_t, and k_d represent the maximum transport rate for saturable transport, the affinity for saturable transport, and the rate constant for nonsaturable transport respectively (Prism v5).

Effects of inhibitors on DNP-SG formation and efflux, total tissue glutathione and GSH/GSSG ratio were analyzed by repeated measures one-way analysis of variance ($\alpha = 0.05$) followed by Dunnett's post-test. Effect of sodium orthovanadate on MTT incorporation was assessed by a paired two-tailed t test ($\alpha = 0.05$) (Prism v5).

ATP-dependent transport of ^{3}H-DNP-SG or ^{3}H-$E_2$17G mediated by different transporters and the effect of inhibitors on MRP1- or MRP2-mediated ^{3}H-DNP-SG transport were compared by one-way ANOVA ($\alpha = 0.05$) followed by Dunnett's post-test. K_t and υ_{max} for MRP1- or MRP2-mediated ATP-dependent ^{3}H-DNP-SG transport were determined by unweighted nonlinear regression and data were fitted to the Michaelis−Menten equation (Prism v5), where, K_t and υ_{max} represent the affinity for saturable transport and the maximum transport rate for saturable transport, respectively.

3. Results

3.1. Human Subjects. Clinical data for patients included in the study are summarized in Table 1. Term placental tissues (≥36 weeks of gestation) were collected from patients undergoing C-section deliveries following normal pregnancies and had no gross abnormalities. Two of the pregnant patients had asthma (patients 4 and 6) and used albuterol on an as needed basis. One patient had polycystic ovarian syndrome (patient 1), another patient had hypothyroidism (patient 5) and used synthroid 125 mg qd. All infants had normal Apgar scores and were healthy.

Table 1. Clinical Data for Patients Undergoing C-Section Delivery Following Normal Pregnancy[a]

maternal age (y)	28.8 ± 3.8 (23−36)
race	
hispanic	3
white	3
black	3
asian	1
gestational age (wk)	38 ± 1 (36−39)
parity	
0	1
1	4
2	5
gravidity	
1	1
2	3
3	6
systolic blood pressure (mmHg)	118 ± 10.7 (103−135)
diastolic blood pressure (mmHg)	71.8 ± 8.0 (60−90)
infant birthweight (kg)	3.36 ± 0.62 (2.81−4.94)
placental weight (g)	731 ± 202 (400−1004)

[a] Total of 10 patients. Data presented as mean ± SD (range), except for race, parity, gravidity.

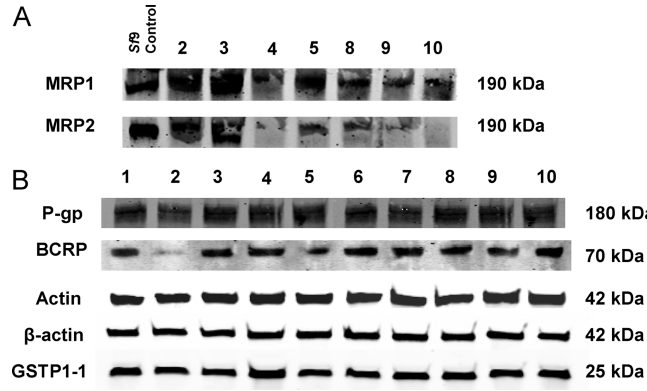

Figure 2. Protein expression in human placental tissue. (A) Western blots illustrating protein expression of MRP1 and MRP2 in MRP1/2-overexpressing membrane vesicles (*Sf*9 protein = 0.05 μg) or tissue homogenates (200 μg homogenate protein/40 μL) of cultured placental tissue from patients 2, 3, 4, 5, 8, 9, 10. (B) Western blots illustrating protein expression of P-gp, BCRP, GSTP1-1 and housekeeping proteins, actin and β-actin in tissue homogenates (50 μg homogenate protein/50 μL) of cultured placental tissue from $n = 10$ patients. Placental tissue from patients 2, 3, 4, 5, 8, 9, 10 was used for inhibition studies described in section 3.4.

3.2. Protein Expression. Protein expression of ABC transporters MRP1 (190 kDa) and MRP2 (190 kDa) was studied in patients 2, 3, 4, 5, 8, 9, 10 (Figure 2A). Protein expression of P-gp (180 kDa) and BCRP (70 kDa), enzyme GSTP1-1 (25 kDa), housekeeping proteins actin (42 kDa) and β-actin (42 kDa) was studied in $n = 10$ patients by Western blotting (Figure 2B). Integrated intensity values corresponding to the protein bands were compared across different patients for each protein (data not shown). Protein

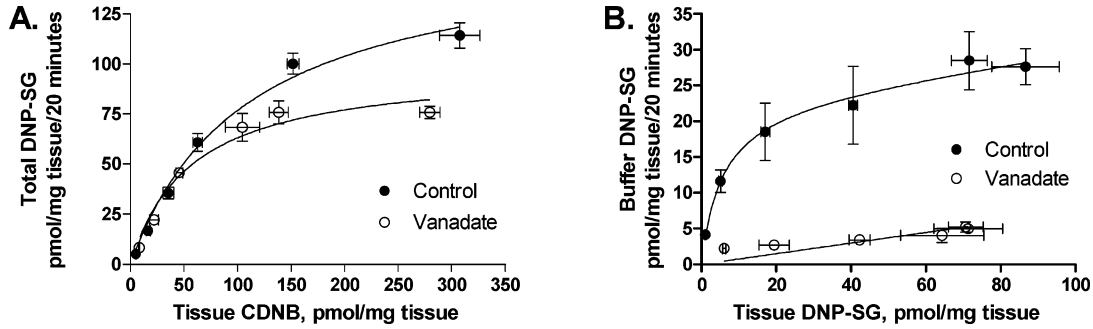

Figure 3. Kinetics of DNP-SG formation and efflux. (A) DNP-SG formation (total DNP-SG) as a function of tissue CDNB content; DNP-SG formation followed classic Michaelis−Menten kinetics both in the absence [K_m =109 (±10) pmol tissue CDNB/mg tissue, v_{max} = 160 (±6) pmol DNP-SG/mg tissue/20 min] and presence of sodium orthovanadate [K_m = 56.7 (±8.1) pmol tissue CDNB/mg tissue, v_{max} = 98.9 (±4.9) pmol DNP-SG/mg tissue/20 min]. (B) DNP-SG efflux as a function of tissue DNP-SG, measured at 20 min upon exposure to 3−1000 μM CDNB in the loading buffer, in the absence (control: closed circles) or presence (vanadate: open circles) of sodium orthovanadate. Data for total transport (control) and diffusional transport (vanadate) were globally fitted to eq 1 with or without the saturable component, respectively (Prism v5). Data represent mean ± SD from triplicate determinations in a representative patient (patient 8). DNP-SG efflux occurred by a combination of passive diffusion [k_d = 0.074 (±0.010)/20 min] and active transport [K_t = 5.5 (±1.3) pmol/mg tissue, J_{max} = 23.1 (±1.4) pmol/mg tissue/20 min].

expression of P-gp, GSTP1-1, actin and β-actin remained unchanged among patients, while the expression of MRP1, MRP2 and BCRP was variable. Of note, MRP2 expression showed large interpatient variability with very low expression in patients 4, 9, and 10. BCRP expression was relatively low in patient 2.

3.3. Kinetics of DP-SG Formation and Efflux. DNP-SG formation followed classic Michaelis−Menten kinetics [K_m =118 (±17) pmol tissue CDNB/mg tissue, v_{max} = 160 (±18) pmol DNP-SG/mg tissue/20 min] while DNP-SG efflux occurred by a combination of passive diffusion [k_d = 0.081 (±0.010)/20 min] and active transport [K_t = 5.3 (±1.6) pmol/mg tissue, J_{max} = 17.3 (±8.2) pmol/mg tissue/20 min] (data represent mean ± SEM from n = 3 patients). Data from a representative patient (patient 8) are illustrated in Figure 3. CDNB loading concentration of 100 μM was determined to be optimum for further experiments. At this CDNB loading concentration formation followed first order kinetics, while DNP-SG efflux was saturated and followed zero order kinetics.

3.4. Effect of GSTP1-1 or ABC Transporter Inhibition on DNP-SG Formation and Efflux. To evaluate the involvement of the enzyme GSTP1-1 and ABC transporters P-gp, BCRP and the MRPs in the formation and efflux of DNP-SG from the human placental villous tissue, the effect of ethacrynic acid (GST inhibitor), sodium orthovanadate (ATPase inhibitor; positive control), MK571 (MRP inhibitor), DP (BCRP/P-gp/MRP1 inhibitor) or verapamil (P-gp inhibitor and MRP1 inhibitor in the presence of glutathione) on DNP-SG formation and efflux was studied.

Treatment with 500 μM ethacrynic acid decreased DNP-SG formation (total DNP-SG) by 78.0 (±10.4)% ($p < 0.05$), but DNP-SG efflux (expressed as a percent of total DNP-SG detected in the efflux buffer) remained unchanged (Figures 4A and 4B). Although the glutathione conjugate of ethacrynic acid (EA-SG) is a known substrate for MRP1 (K_m = 5−28 μM),[44,45] DNP-SG efflux was not inhibited in its presence. Furthermore, ethacrynic acid treatment inhibited DNP-SG formation in a concentration-dependent manner in the concentration range of 0.15−1.5 mM, whereas DNP-SG efflux was not affected.[30]

DNP-SG efflux decreased by 69.1 (±11.3)%, 51.1 (±5.4)%, 56.7 (±8.3)% and 53.6 (±10.8)% ($p < 0.05$) in the presence of 5 mM sodium orthovanadate, 100 μM MK571, 1 mM DP and 100 μM verapamil respectively, upon exposure to 100 μM CDNB (Figure 4D, 4F). DNP-SG formation decreased marginally by 20.7 (±7.6)% ($p < 0.05$) in the presence of 5 mM sodium orthovanadate, but remained unchanged in the presence of MK571, DP and verapamil (Figure 4C, 4E). Furthermore, DNP-SG efflux was inhibited in a concentration-dependent manner by MK571, DP and verapamil in the concentration ranges of 30−300 μM, 0.3−3 mM and 30−300 μM, respectively, while DNP-SG formation was not affected (data not shown).

3.5. Effect of GSTP1-1 or ABC Transporter Inhibition on Biochemical Parameters. To ascertain that the changes observed in DNP-SG efflux upon treatment with ABC transporter inhibitors were due to inhibition of ABC transporter activity and not due to biochemical changes such as loss of tissue integrity, reduction in tissue viability or changes in the tissue redox status, LDH release into the media was measured as a marker for tissue integrity, MTT incorporation was measured as a marker for tissue viability and the GSH/GSSG ratio was determined to monitor the

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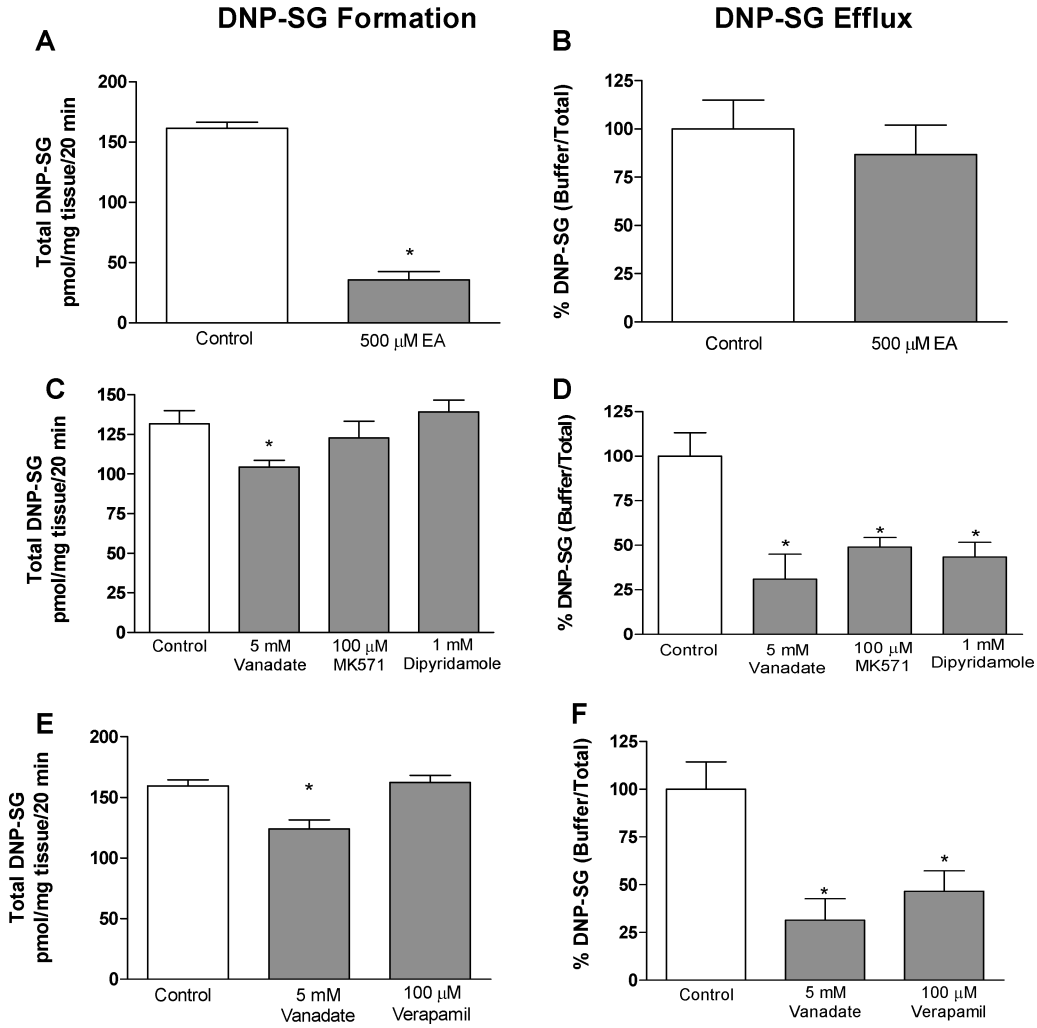

Figure 4. Effect of GSTP1-1 or ABC transporter inhibition on DNP-SG formation or efflux. DNP-SG formation (A, C and E) and efflux (B, D and F) in the absence (control) or presence of inhibitors upon exposure to 100 μM CDNB. Data represent mean $\pm$ SEM from triplicate determinations in $n = 6$ patients. * indicates $p < 0.05$ vs control when compared by paired two-tailed *t*-test (A) or by repeated measures one-way ANOVA followed by Dunnett's post-test (C, D, E, F). Control values: DNP-SG efflux (buffer/total) ratio. B: 0.36 [95% CI: (0.32,0.40)], experiment done 6 h post-culture (patients 3, 4, 5, 8, 9, 10). D: 0.30 [95% CI: (0.27,0.33)], experiment done 10 h post-culture (patients 2, 4, 5, 8, 9, 10). F: 0.36 [95% CI: (0.32,0.40)], experiment done 6 h post-culture (patients 2, 3, 4, 8, 9, 10).

redox status of the tissue. LDH was not detected in any of the media samples, after 1 h tissue incubation with or without the various inhibitors, indicating that treatment with inhibitors did not affect the tissue integrity. Furthermore, as shown in Figure 5A, MTT incorporation remained unchanged in tissue samples treated without or with ATPase inhibitor sodium orthovanadate (5 mM), suggesting that the tissue viability was also maintained. MTT incorporation was not studied in tissue samples treated with the other ABC transporters used in this study, however, these inhibitors are likely to affect tissue integrity to a lesser extent than sodium orthovanadate. Sodium orthovanadate inhibits all the ATP dependent processes in the tissue and is expected to have the most drastic adverse effect on tissue viability.

Total tissue glutathione content (Figure 5B) and GSH/GSSG ratios (Figure 5C) were compared between samples treated with or without various inhibitors. Depletion of tissue glutathione might lead to a decrease in DNP-SG formation whereas a decrease in the GSH/GSSG ratio would indicate increased accumulation of GSSG, suggesting that the tissue might be under oxidative stress. No changes were observed in the total glutathione content or the GSH/GSSG ratio upon treatment with ABC transporter inhibitors: 5 mM sodium orthovanadate, 100 μM verapamil and 100 μM MK571. Total glutathione and GSH/GSSG ratio were not monitored for DP ($\lambda_{max} = 420$ nm) due to spectroscopic interferences in the glutathione assay monitored at $\lambda = 405$ nm. Upon treatment with GST inhibitor ethacrynic acid, total glutathione content (Figure 4B) decreased by 0.180 [95% CI: 0.034−0.330] μmol/g wet tissue, but the GSH/GSSG ratio remained unchanged. Ethacrynic acid itself undergoes glutathione conjugation leading to depletion of tissue glutathione, and also acts by inhibiting the placental GSTP1-1 enzyme. Decreased total tissue glutathione content and the observed decrease in DNP-SG formation upon treatment with

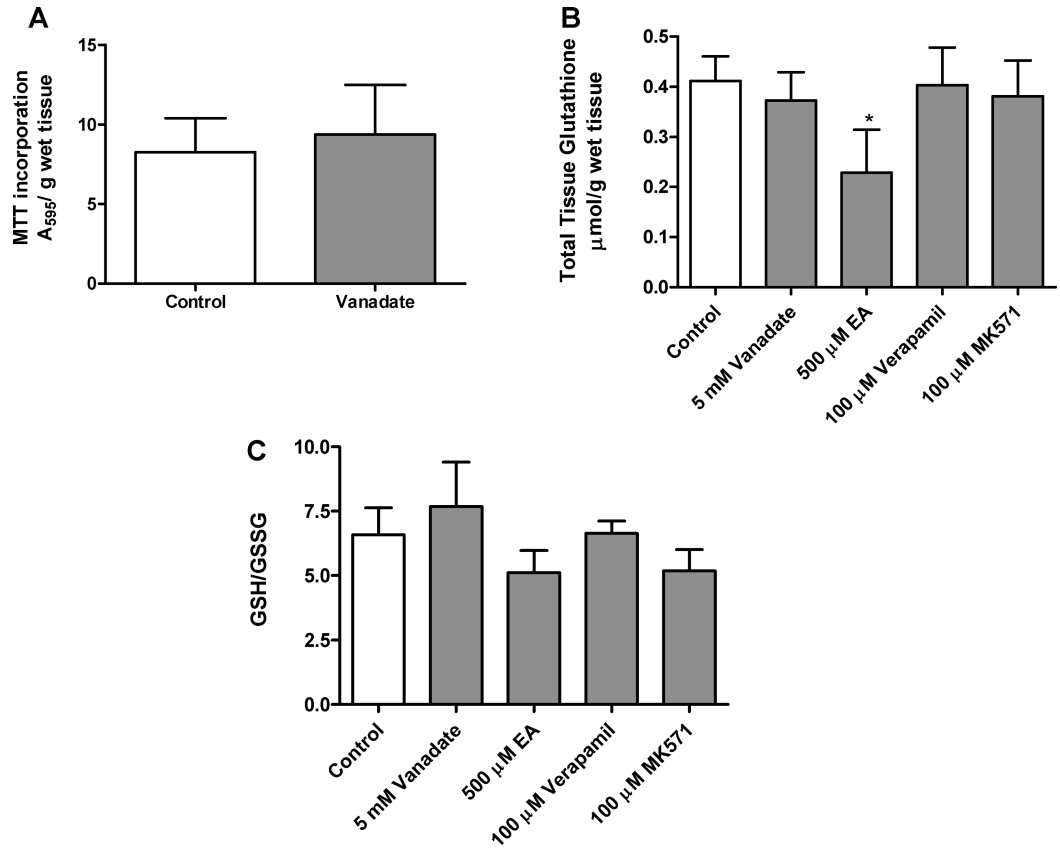

Figure 5. Effect of inhibitors on biochemical markers. Effect of GSTP1-1 or ABC transporter inhibitors on MTT incorporation - marker for tissue viability (A), total tissue glutathione content (B) and ratio of reduced to oxidized glutathione (GSH/GSSG) - marker for tissue redox status (C) after 1 h tissue incubation in the absence (control) or presence of inhibitors. Data represent mean ± SEM from triplicate determinations in $n = 3$ patients. * indicates $p < 0.05$ vs control when compared by repeated measures one-way ANOVA followed by Dunnett's post-test. MTT incorporation was measured 48 h post-culture (patients 6, 8, 9), and total glutathione and GSH/GSSG ratios were measured between 6 and 10 h post-culture (patients 6, 9, 10).

ethacrynic acid are consistent with these mechanisms of inhibition previously described for ethacrynic acid.[35,46]

3.6. ^{3}H-DNP-SG or ^{3}H-$E_2$17G Transport into Inside-Out *Sf*9 Membrane Vesicles. DNP-SG is transported by the MRPs and BCRP;[16−19,27] however data are lacking about its interaction with P-gp. To assess if DNP-SG is a substrate for P-gp and to determine the relative transport rates for ATP-dependent transport mediated by the different transporters, ^{3}H-DNP-SG transport (10 μg of protein, 5 min incubation) was measured in membrane vesicles individually overexpressing P-gp, BCRP, MRP1, MRP2, or MRP3 and in the control membrane vesicles. As a positive control for functional activity of the various transporters, ^{3}H-$E_2$17G transport was also determined in the same set of membrane vesicles. $E_2$17G was chosen since it is a known substrate for P-gp, BCRP as well as MRP1, MRP2 and MRP3.[19,43,47−49] At a ^{3}H-DNP-SG concentration of 60 nM, MRP1- or MRP2-mediated ATP-dependent ^{3}H-DNP-SG transport was greater than MRP3-, BCRP- or P-gp-mediated transport (Figure 6A). MRP1-mediated ^{3}H-DNP-SG transport (792 ± 85 fmol/mg protein/min) was 2-fold higher ($p < 0.05$) than MRP2-mediated transport (393 ± 36 fmol/mg protein/min). ATP-dependent $E_2$17G transport (49.6 nM) was observed in all the membrane vesicles (Figure 6B) and was significantly greater ($p < 0.05$) than transport observed in the EV membrane vesicles.

3.7. Optimization of ^{3}H-DNP-SG Transport Activity Measurements. The transport of ^{3}H-DNP-SG into MRP1- or MRP2-expressing membranes was ATP-dependent, increased linearly with time up to 10 min and was much greater in membrane vesicles expressing MRP1 or MRP2 versus EV. The time course of BCRP-mediated ATP-dependent ^{3}H-DNP-SG transport was also assessed. BCRP-mediated ^{3}H-DNP-SG transport was ATP-dependent and was about 2-fold greater than that in membrane vesicles expressing EV (Figure 7). However, this small difference was not consistently observed in different experiments.

ATP-dependent ^{3}H-DNP-SG transport into MRP2-, BCRP- or EV-expressing membrane vesicles was linear with respect to protein concentration and occurred into an osmotically

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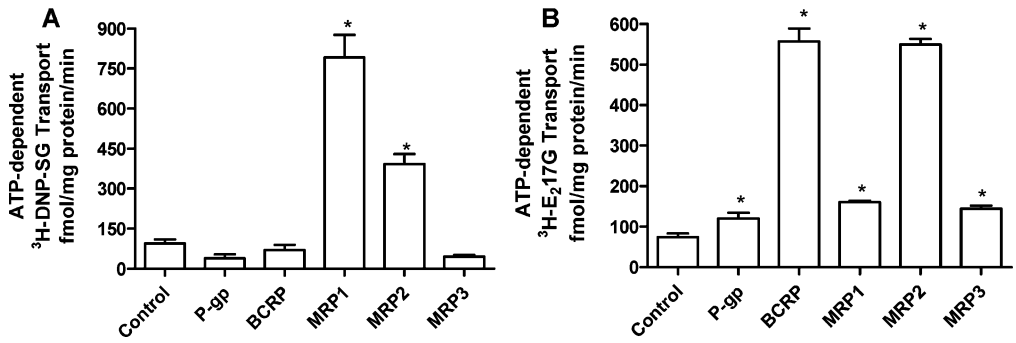

Figure 6. ^{3}H-DNP-SG or ^{3}H-E$_2$17G transport in *Sf*9 membrane vesicles. ATP-dependent ^{3}H-DNP-SG (60 nM) (A) or ^{3}H-E$_2$17G (49.6 nM) (B) transport activity in membrane vesicles infected with empty virus (control), or expressing P-gp, BCRP, MRP1, MRP2, or MRP3 (10 μg protein, 5 min incubation time). Data represent mean ± SD from $n = 3$ determinations. * represents $p < 0.05$ vs control when compared by one-way ANOVA followed by Dunnett's post-test.

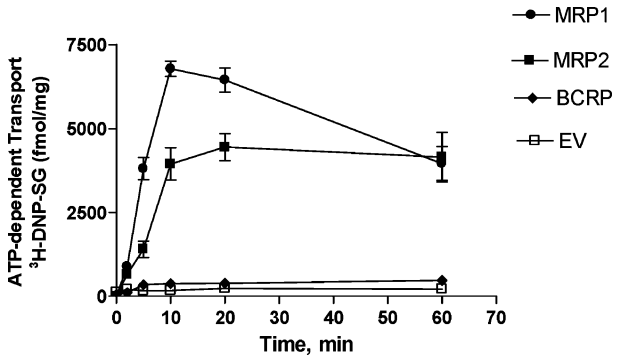

Figure 7. Time course of ^{3}H-DNP-SG transport in *Sf*9 membrane vesicles. Time course of ATP-dependent ^{3}H-DNP-SG (60 nM) transport activity in *Sf*9 membrane vesicles (10 μg protein) infected with EV (control), or expressing BCRP, MRP1 or MRP2. Data represent mean ± SD from $n = 3$ determinations.

sensitive space (data not shown). These data suggest that the observed activity was mainly transport and that binding to the membrane vesicles was minimal. Based on the results of these initial experiments, an incubation time of 5 min and *Sf*9 protein mass of 10 μg were chosen as optimal conditions for further experiments to characterize MRP1- or MRP2-mediated ^{3}H-DNP-SG transport. ATP-dependent transport of ^{3}H-DNP-SG was quite low in the BCRP-overexpressing *Sf*9 membrane vesicles (Figure 7), hence the kinetics or the effects of inhibitors on BCRP-mediated ATP-dependent ^{3}H-DNP-SG transport were not characterized.

3.8. MRP1- or MRP2-Mediated ^{3}H-DNP-SG Transport. ATP-dependent ^{3}H-DNP-SG transport was saturable in both MRP1- and MRP2-expressing membrane vesicles and was best fit to the Michaelis−Menten equation (Figure 8A). MRP1-mediated ^{3}H-DNP-SG transport [$K_t = 11.3 \pm 1.3$ μM and $v_{max} = 86.7 \pm 1.9$ pmol/mg/min] was a relatively high-affinity, low-capacity process whereas MRP2-mediated ^{3}H-DNP-SG transport [$K_t = 168 \pm 7$ μM and $v_{max} = 1367 \pm 19$ pmol/mg/min] was a relatively low-affinity, high-capacity process.

To assess if there are any differences in the interaction of MRP1 and MRP2 with the inhibitors that were used for the placental villous tissue experiments, MRP1- and MRP2-mediated ^{3}H-DNP-SG transport rates were measured in the presence of sodium orthovanadate (0.5 mM), MK571 (100 μM), DP (100 μM) and verapamil (100 μM), in the presence or absence of 0.5 mM glutathione. MRP1-mediated ^{3}H-DNP-SG transport (Figure 8B) showed a 34.3 (±5.9)%, 99.8 (±0.3)%, 21.9 (±12.1)% and 99.1 (±3.4)% decrease in the presence of sodium orthovanadate, MK571, DP and verapamil in the presence of glutathione, respectively ($p < 0.05$). MRP2-mediated ^{3}H-DNP-SG transport (Figure 8C) showed a 38.9 (±4.9)% and 89.3 (±1.5)% decrease in the presence of sodium orthovanadate and MK571 respectively ($p < 0.05$), but showed a 193 (±12)% and 139 (±13)% increase in the presence of DP and verapamil respectively ($p < 0.05$), and was not affected by the presence or absence of glutathione.

4. Discussion

4.1. Effect of Inhibitors on DNP-SG Formation and Efflux in Human Placental Villous Tissue. DNP-SG efflux was inhibited to approximately the same extent in the presence of sodium orthovanadate, MK571, dipyridamole and verapamil, while DNP-SG formation or tissue biochemical parameters were not affected (Figures 4, 5). These observations suggest nonspecific inhibition of all ATP-dependent processes by these inhibitors, and that one or more of the apical ABC transporters might be involved in DNP-SG efflux. Alternatively, a single transporter such as MRP1 that might have been inhibited to the same extent by all these inhibitors might play a major role in DNP-SG efflux. Complete inhibition of DNP-SG was not observed in the placental villous tissue, since DNP-SG efflux in the villous tissue occurs by a combination of passive diffusion and active transport. Furthermore, inhibitor concentrations used for the placental villous tissue studies did not reflect the actual unbound intracellular inhibitor concentrations at the site of the transporters. In view of this limitation, kinetic characterization of the inhibition process was not possible.

In mammalian tissue systems such as the placental villous tissue explants model, functional characterization of individual transporters is difficult due to the lack of selective substrates and/or specific inhibitors, as well as the presence of compensatory regulatory mechanisms. Furthermore, cotrans-

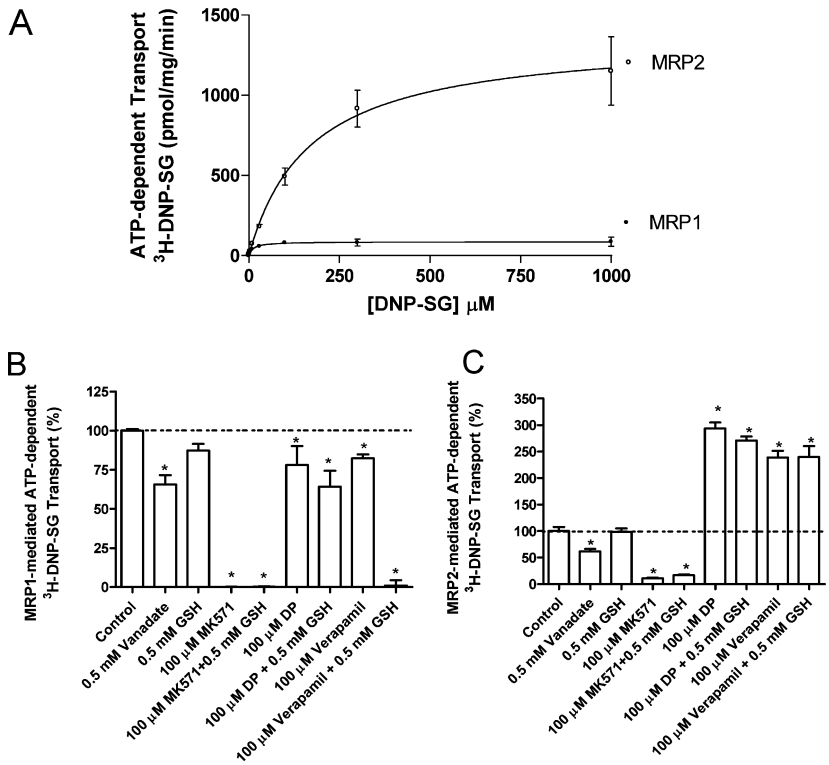

Figure 8. MRP1- or MRP2-mediated ^{3}H-DNP-SG Transport. Saturation (A) or effect of inhibitors (B and C) of MRP1/MRP2-mediated ATP-dependent ^{3}H-DNP-SG (60 nM) transport activity in *Sf*9 membrane vesicles expressing MRP1 or MRP2 (10 μg protein, 5 min incubation time). Data represent mean $\pm$ SD from $n = 3$ determinations. A: Nonlinear regression was performed by fitting the unweighted data to the Michaelis−Menten equation. B and C: Transport activity in MRP1 (B) or MRP2 (C) expressing membrane vesicles in the absence (control) or presence of inhibitors (with or without glutathione). Control values: MRP1-mediated transport, 792 $\pm$ 85 fmol/mg protein/min; MRP2-mediated transport, 393 $\pm$ 36 fmol/mg protein/min (10 μg protein, 5 min incubation time). * represents $p < 0.05$ vs control when compared by one-way ANOVA followed by Dunnett's post-test.

port mechanisms and the presence of multiple binding sites have been investigated for the MRP transporters and other ABC transporters.[50] In view of these challenges, *in vitro* transport measurements were performed in *Sf*9 membrane vesicles to enable assessment of the interaction between individual transporters, substrates and inhibitors.

4.2. Role of MRP1 and Other ABC Transporters in DNP-SG Efflux. MRP1-mediated ^{3}H-DNP-SG transport in the MRP1-overexpressing *Sf*9 membrane vesicles was inhibited by sodium orthovanadate, MK571, DP and verapamil in the presence of glutathione. MRP1-mediated ^{3}H-DNP-SG transport in *Sf*9 membrane vesicles had an apparent affinity ($K_t = 11.3 \pm 1.3$ μM) similar to that observed for DNP-SG efflux from the villous tissue ($K_t = 5.3$ μM) as well as previously reported estimates from MRP1-mediated DNP-SG transport in plasma membrane vesicles from HeLa T5 cells.[17] The kinetics and the inhibition pattern observed for MRP1-mediated ^{3}H-DNP-SG transport were similar to those observed in the villous tissue fragments, suggesting that MRP1 may play a major role in the efflux of DNP-SG from the human placental villous tissue.

The involvement of other ABC transporters including P-gp, BCRP and MRP3 in the efflux of DNP-SG cannot be ruled out, but is unlikely since MRP1 transports DNP-SG more efficiently (Figure 6A) than these other ABC transporters and is highly expressed in the human placenta compared to other tissues.[2,51] Although experiments were performed at a single DNP-SG concentration (60 nM), these data are in agreement with the previously reported interaction of DNP-SG with ABC transporters. There is no reported

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evidence of P-gp-mediated DNP-SG transport, and we did not detect this activity. In addition, data available for interaction of DNP-SG with BCRP or MRP3 suggest that these transporters are less active in ATP-dependent DNP-SG transport.[27,52]

MRP2-mediated DNP-SG transport is likely, since DNP-SG is a well-established substrate for MRP2.[17,48] However, MRP2 expression in the human placenta is relatively low.[53] Furthermore, compared to MRP1, MRP2-mediated DNP-SG transport was found to be a low-affinity process [$K_t = 168 \pm 7$ μM]. The parameter estimates for the affinity of MRP2 toward DNP-SG transport were comparable with previously reported estimates from MRP2-mediated DNP-SG transport in human canalicular membrane vesicles ($K_t = 190$ μM)[54] but differed from those observed for DNP-SG efflux from the villous tissue ($K_t = 5.3$ μM). The maximum transport rate for saturable transport (v_{max}) could not be compared across the different systems due to expected difference in protein expression of MRP2 in the placental villous tissue or *Sf*9 membrane vesicles or the human canalicular membrane vesicles. Furthermore, MRP2-mediated DNP-SG transport was inhibited by sodium orthovanadate and MK571 but unlike MRP1 it was stimulated by 193 (±12)% and 139 (±13)% in the presence of verapamil (100 μM) and DP (100 μM) respectively. The inhibition pattern or the kinetic characteristics of MRP2 do not match the observations related to DNP-SG efflux from the human placenta, suggesting that MRP2 might not have a major role in DNP-SG efflux from the placental tissue.

4.3. Effect of Inhibitors on MRP1- or MRP2-Mediated DNP-SG Transport. Inhibition of both MRP1 and MRP2-mediated DNP-SG transport by sodium orthovanadate and MK571 was expected since their roles as ATPase inhibitor and MRP-family inhibitor, respectively, have been well established. We observed modest inhibition of MRP1-mediated DNP-SG efflux in the presence of 100 μM DP, which was further enhanced in the presence of 0.5 mM glutathione (Figure 8B), suggesting that cellular glutathione content may have an important role in the DP-mediated inhibition of MRP1. However, MRP2-mediated DNP-SG transport was stimulated in the presence of DP and this process did not appear to be affected by the presence of glutathione (Figure 8C). Further studies are required to understand the mechanisms for inhibition of MRP1 and stimulation of MRP2-mediated transport in the presence of 100 μM DP. Additionally, we observed a modest inhibition of MRP1-mediated DNP-SG transport in the presence of verapamil, but in the presence of verapamil and glutathione, MRP1-mediated DNP-SG efflux was completely inhibited (Figure 8B). Loe et al. reported stimulation of MRP1-mediated glutathione efflux in the presence of verapamil.[34] Inhibition of MRP1-mediated efflux in the presence of glutathione, but not in the absence of glutathione may be consistent with competitive inhibition of DNP-SG efflux due to stimulation of GSH efflux in the presence of verapamil. The cultured human placental villous tissue contains glutathione (0.4−0.5 mM) (Figure 5B) as previously reported,[55] and verapamil induced stimulation of glutathione efflux would explain the inhibition of DNP-SG efflux in the human placental villous tissue model as well. Unlike MRP1, MRP2-mediated DNP-SG transport was stimulated in the presence of verapamil, and this process was not affected by glutathione (Figure 8C). This observation was not consistent with verapamil mediated inhibition of DNP-SG efflux in the villous tissue. The mechanism for verapamil-mediated stimulation of MRP2-mediated DNP-SG efflux is not clear, but argues against an apparent role for MRP2 in the placental efflux of DNP-SG. Instead, our data support a role for either MRP1 or an MRP1-like transporter.

4.4. Localization of MRP1 in the Placental Syncytiotrophoblast. There are contradicting reports about the localization of MRP1 on the placental syncytiotrophoblast in the literature. St-Pierre et al.[2] first reported that MRP1 is located on the apical side of the placental syncytiotrophoblast as well as on the fetal capillary endothelium, and is likely to play a protective role by promoting efflux in the fetal to maternal direction (Figure 1). In contrast, Atkinson et al. and Nagashige et al. reported a basolateral localization for MRP1 on the placental syncytiotrophoblast opposing the maternally facing MRP1 on the fetal capillary endothelium. This suggests that compounds that are effluxed by MRP1 in the placenta would accumulate in the placental villous connective tissue.[56,57] Results from the present study indicate the involvement of an apical ATP-binding cassette transporter that is inhibited by MK571, DP and by verapamil in the efflux of DNP-SG from the villous tissue. This inhibition pattern is consistent with MRP1-mediated transport activity and was confirmed by ^{3}H-DNP-SG transport data from *Sf*9 membrane vesicle experiments. In the cultured placental villous tissue model, the fetal capillaries are collapsed and

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basolateral DNP-SG efflux would not be expected from this system.[3,58] Furthermore, histological examination of the villous tissue revealed that the fetal capillary endothelial vessels are not exposed to the medium,[30] hence the observed DNP-SG efflux cannot be attributed to maternally directed efflux by MRP1 localized on the abluminal side of the fetal capillary endothelium. The suggested apical localization of MRP1 in the placental syncytiotrophoblast differs from the basolateral localization of this transporter in hepatocytes[59] but is consistent with its apical localization at the blood−tissue barriers in other sanctuary organs or protected compartments such as the brain capillary endothelial cells.[60−62]

4.5. Role of MRP1 in Disposition. Drugs that are substrates for MRP1 include HIV protease inhibitors (ritonavir and saquinavir) as well as anticancer agents (vinblastine, vincristine, doxorubicin, daunorubicin, etoposide, and methotrexate).[63] The HIV protease inhibitor combination of lopinavir and ritonavir is used as a part of the highly active antiretroviral therapy (HAART) in pregnant patients to prevent materno−fetal transmission of the HIV virus, and the intention of treatment is to achieve therapeutic concentrations of these antiviral agents in the fetus.[64] Efflux by MRP1 in the feto−maternal direction is likely to decrease the degree of materno−fetal transfer of HIV protease inhibitors used in pregnancy.

In addition to transport of these drugs, MRP1 also transports hydrophilic anionic substrates including glutathione, glucuronide, sulfate conjugates of xenobiotics or endogenous substances like bile acids and steroids, as well as other substrates such as bilirubin glucuronides, leukotrienes and unconjugated bilirubin.[17,68] St-Pierre et al. reported ATP-dependent uptake of ^{14}C-DNP-SG and $E_2$17G in inside-out human term placental apical membrane vesicles;[2] however further data regarding the functional activity of MRPs in the human placenta are lacking. Pascolo et al. reported the involvement of MRP1 in the efflux of unconjugated bilirubin from the apical membrane of polarized BeWo cells, suggesting that MRP1 located on the apical side of trophoblast cells might play an important role in protecting the fetus from potentially toxic metabolites such as unconjugated bilirubin.[69] It has been suggested that the human placenta maintains a feto−maternal gradient of conjugated and unconjugated bilirubin as well as bile acids, in both normal and cholestatic pregnancies.[4]

The human placental expression and activity of GSTP1-1[28] and UDP-glucuronosyltransferases (UGT2B4 and UGT2B7),[65] sulfotransferases and other phase II conjugative enzymes have been established,[66] and these enzymes may protect the fetus by forming conjugates that would be effluxed by MRP1. Glutathione and glutathione dependent enzymes such as GSTs play an important role in detoxification of xenobiotics and may offer protection against oxidative stress. MRP1 is involved in the efflux of glutathione conjugates as well as GSSG,[17] and may play an important role in maintaining the GSH/GSSG ratio in response to oxidative stress. Certain diseases of pregnancy such as preeclampsia and gestational diabetes are associated with oxidative stress,[67] and might also be associated with changes in expression or activity of transporters such as MRP1 which play an important role in maintaining the GSH/GSSG ratio. Furthermore, since MRP1 pumps the glutathione conjugate of the lipid peroxidation product 4-hydroxynonenal out of cells,[70] its activity in the placenta may be important in the elimination of this reactive conjugate from placental tissue under conditions of oxidative stress.

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5. Conclusion

The ^{3}H-DNP-SG transport data in *Sf*9 membrane vesicles support the findings from the human placental villous tissue experiments, and suggest that CDNB is detoxified from the human placental villous tissue by the coordinated actions of GSTP1-1 and an apical ABC transporter such as MRP1. The involvement of other transporters such as MRP2 or BCRP in DNP-SG efflux cannot be ruled out but is likely to be less important. The localization of MRP1 in the placental syncytiotrophoblast is somewhat uncertain; however the data presented herein provide support for an apical localization for MRP1 or an MRP1-like transporter.

Abbreviations Used

ABC, ATP-binding cassette; BCRP, breast cancer resistance protein; CDNB, 1-chloro-2,4-dinitrobenzene; DNP-SG, 2,4-dinitrophenyl-*S*-glutathione; DP, dipyridamole; DPBS, Dulbecco's phosphate buffered saline; $E_2$17G, estradiol 17-(β-D-glucuronide); EA, ethacrynic acid; FDNB, 1-fluoro-2,4-dinitrobenzene; LDH, lactate dehydrogenase; LLOQ, lower limit of quantitation; GSH, reduced glutathione; GSSG, oxidized glutathione; GSTP1-1, glutathione-*S*-transferase isoform P1-1; MRP, multidrug resistance associated protein; MTT, thiazolyl blue tetrazolium bromide; P-gp, P-glycoprotein; PCA, perchloric acid.

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