"Inside-Out" Membrane Vesicles: an *in vitro* Model to Study Transporter-mediated Drug Interactions that can Lead to Liver Toxicity

CORNING

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Abstract

Multidrug resistance proteins (MRPs), MRP2 and the bile salt export pump (BSEP) in particular, are the two major ABC efflux transporters that contribute to drug-induced liver toxicity. MRP2 is essential for hepatobiliary elimination of many drugs, drug conjugates, and endogenous compounds, such as bilirubin glucuronides. MRP2 dysfunction caused by inherited MRP2 deficiency or drug-mediated inhibition results in hyperbilirubinemia. BSEP is the rate-limiting step of bile salt transport across hepatocyte membranes. Disruption of BSEP in hepatocytes leads to accumulation of cytotoxic bile salts in the liver, resulting in liver toxicity (e.g., cholestasis). In light of the evidence showing the importance of MRP2 and BSEP in drug-induced liver toxicity, the International Transporter Consortium (ITC) recommends evaluating the potential interaction of NMEs (new molecular entities) with MRP2 and BSEP. In addition, other ABC transporters, i.e., BCRP, localized on the canalicular membrane of hepatocytes are the target of drug-drug interaction (DDI). This study focused on the use of "inside-out" membrane vesicles, which are prepared from insect cells (Sf9) infected with baculovirus expressing human MRP2, BSEP, or BCRP cDNA, to access drug interactions for these transporters *in vitro*. The data demonstrate that in the presence of ATP, MRP2 vesicles showed significant uptake (~10-fold) for leukotriene C4 (LTC4) and estradiol-17β-glucuronide, known MRP2 substrates, compared to control samples (vesicles assayed with the non-hydrolyzable ATP analog AMP). Taurocholate, a BSEP probe substrate, showed more than 20-fold uptake activity in BSEP vesicles, compared to the control. MRP2-mediated uptake of the probe substrate LTC4 was significantly inhibited by known MRP2 modulators (e.g., MK571, benzbromarone, terfenadine, and indomethacin). BSEP-mediated taurocholate uptake was shown to be inhibited by known BSEP inhibitors (e.g., cyclosporine A, glibenclamide, troglitazone, etc). BCRP-mediated estrone-3-sulfate was inhibited by BCRP modulators (e.g., sulfasalazine, novobiocin, FTC, and Ko143). The data indicate that "inside-out" membrane vesicles are a quick and useful tool to screen MRP2, BSEP, and BCRP inhibitors that can potentially cause liver toxicity or DDI *in vivo*.

Introduction

The ATP binding cassette (ABC) family is a multi-transmembrane protein that plays a vital role in drug absorption, distribution, elimination, and toxicity. Inverted plasma membrane vesicles prepared from transporter over-expressed cells are increasingly used to study ABC efflux transport.[1,2] The advantage of this model versus whole cell model is that the test articles do not need to be preloaded into the cells. The vesicle models work for both permeable and poorly permeable drugs. In this study, we present the application of inside-out vesicles in characterizing ABC transporter substrate specificity and drug inhibitory effect on transporter-mediated drug excretion.

Figure 1: Workflow of vesicle uptake assay

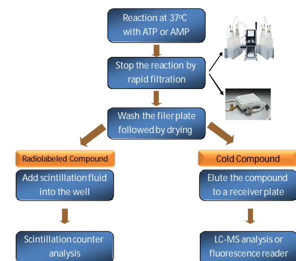


Figure 1: Workflow of vesicle uptake assay. After incubating the test articles with vesicles, the assay is terminated by rapid filtration using a cell harvester or vacuum manifold. After washes, the radiolabeled compound is ready to read. For native compounds, add elution buffer to the filter plate and elute the compound to a receiver plate for subsequent analysis.

Materials and Methods

Materials

Corning® Gentest™ human MRP2 (Cat. No. 453450); human BCRP (Cat. No. 453271) and human BSEP (Cat. No 453502) vesicles: Corning Gentest MRP/BCRP Vesicle Assay Kit (Cat. No. 459010) and BSEP Vesicle Assay Kit (Cat. No. 459011): 10 X Wash Buffer for MRP/BCRP Vesicle Assay (Cat. No. 450600) and 10 X Wash Buffer for BSEP Vesicle Assay (Cat. No. 450601).

Transport Uptake Assay

The uptake was carried out using a rapid filtration system (vacuum manifold). A 60 µl reaction mixture containing 50 µg vesicles, 2.5 mM GSH (only used for MRP2) and probe substrate or test compounds in uptake buffer was pre-incubated at 37°C for 5 minutes. For inhibitory studies, test inhibitor were also pre-incubated in uptake buffer with 50 µg vesicles and substrates for 5 min. The uptake was initiated by addition of 5 mM ATP or AMP, followed by incubation at 37°C. The assay was terminated by rapid filtration followed by washing with cold washing buffer. After filter is dry, it is ready for analysis by scintillation counter or LC-MS following the manufacture protocol.

Data Analysis

ATP-dependent uptake activity was reported as uptake activity in the presence of ATP subtracting uptake activity in the presence of AMP. The percentage of remaining activity was reported as the uptake activity in the presence of inhibitor divided by the uptake activity in the absence of inhibitor. SigmaPlot™ software was used to analyze the kinetics data. IC_{50} was calculated by linear interpolation using the following equation:

$$IC_{50} = \frac{(50\% - \text{Low \% Inhibition})}{(\text{High \% inhibition} - \text{Low \% Inhibition})} \times (\text{High Con.} - \text{Low Con.}) + \text{Low Con.}$$

Reference

1. Hillgren KM, et al., (2013) Clinical Pharmacology & Therapeutics 94(1):52-63
2. Brouwer K, et al., (2013) Clinical Pharmacology & Therapeutics 94(1):95-112

Figure 2: Product Performance and Lot-to-Lot Consistency

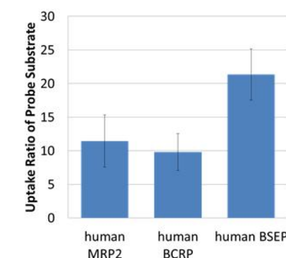


Figure 2: Lot-to-lot consistency for uptake ratio. Three recent lots of human MRP2, BCRP and BSEP vesicles were incubated with 0.1 µM LTC4, 1 µM E3S, and 1 µM of TCA at 37°C in the presence of 5 mM ATP or AMP for 4 min, 4 min, and 30 min, respectively. The results represent the mean of the uptake ratio of the indicated probe substrates in three lots of vesicle products ± S.D..

Figure 3: Time-dependent Uptake of Probe Substrate for MRP2, BCRP, and BSEP Vesicles

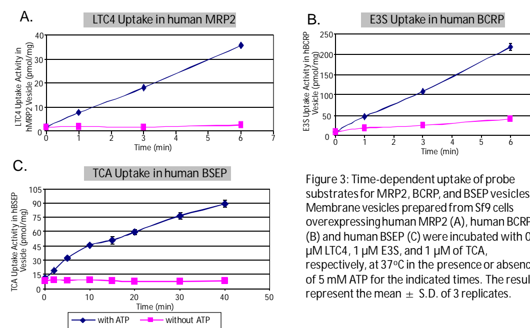


Figure 3: Time-dependent uptake of probe substrates for MRP2, BCRP, and BSEP vesicles. Membrane vesicles prepared from Sf9 cells overexpressing human MRP2 (A), human BCRP (B) and human BSEP (C) were incubated with 0.1 µM LTC4, 1 µM E3S, and 1 µM of TCA, respectively, at 37°C in the presence or absence of 5 mM ATP for the indicated times. The results represent the mean ± S.D. of 3 replicates.

Figure 4. Kinetic Parameters of Probe Substrate Uptake Activity for MRP2, BCRP, and BSEP Transporter Vesicles

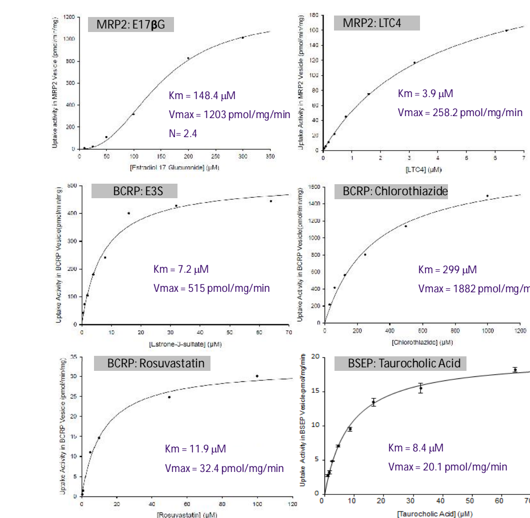
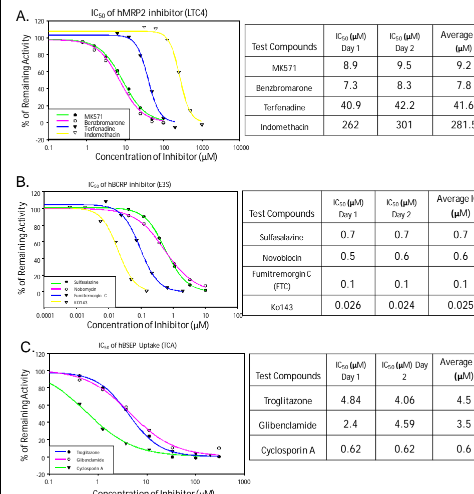


Figure 4: Kinetic Analysis. Uptake of LTC4 and Estradiol-17β-glucuronide in human MRP2 vesicles, uptake of Estrone-3-sulfate, Chlorothiazide and Rosuvastatin in human BCRP vesicle, and uptake of Taurocholic acid in human BSEP vesicles were examined at various concentration in the presence of ATP or AMP. Y axis represents ATP-dependent uptake activity which was calculated by using the uptake activity in the presence of ATP minus uptake activity in the presence of AMP. K_m and V_{max} was calculated using SigmaPlot™ and shown as insert in the graph.

Figure 5. IC_{50} Determination of MRP2, BCRP, and BSEP Transporter Inhibitors



A.

Test Compounds	IC_{50} (µM) Day 1	IC_{50} (µM) Day 2	Average IC_{50} (µM)
MK571	8.9	9.5	9.2
Benzbromarone	7.3	8.3	7.8
Terfenadine	40.9	42.2	41.6
Indomethacin	262	301	281.5

B.

Test Compounds	IC_{50} (µM) Day 1	IC_{50} (µM) Day 2	Average IC_{50} (µM)
Sulfasalazine	0.7	0.7	0.7
Novobiocin	0.5	0.6	0.6
Fumitremorgin C (FTC)	0.1	0.1	0.1
Ko143	0.026	0.024	0.025

C.

Test Compounds	IC_{50} (µM) Day 1	IC_{50} (µM) Day 2	Average IC_{50} (µM)
Troglitazone	4.84	4.06	4.5
Glibenclamide	2.4	4.59	3.5
Cyclosporin A	0.62	0.62	0.6

Figure 5: Inhibitory effects of selected transporter modulators on probe substrates uptake in human MRP2, BCRP, and BSEP membrane vesicles. Human MRP2 (A), BCRP (B), and BSEP (C) vesicles were incubated in assay buffer containing 0.1 µM LTC4, 1 µM E3S, and 1 µM TCA, respectively, and inhibitors at indicated concentration in the presence of ATP or AMP. IC_{50} assays were performed on two independent days. Curves are representative study for each transporter.

Figure 6. Comparison of IC_{50}: LC-MS/MS vs. Radiolabeled Compound

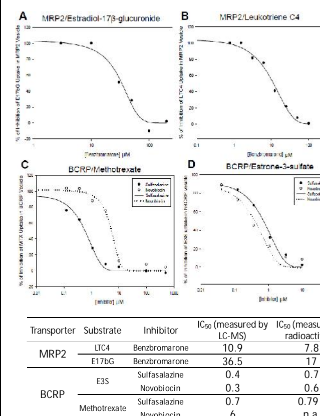


Figure 6: LC-MS/MS application for IC_{50} assay. 80% Methanol was applied to each sample well on the filter plate during the elution step. The plate was incubated with elution buffer for 20 min at room temperature, followed by centrifugation at 4,000 rpm for 20 min. The eluent was collected in a 96 well receiver plate. Curves are representative of two independent IC_{50} assays.

Transporter	Substrate	Inhibitor	IC_{50} (measured by LC-MS)	IC_{50} (measured by radioactivity)
MRP2	LTC4	Benzbromarone	10.9	7.8
	E17bG	Benzbromarone	36.5	17
BCRP	E3S	Sulfasalazine	0.4	0.7
		Novobiocin	0.3	0.6
	Methotrexate	Sulfasalazine	0.7	0.79 *
		Novobiocin	6	n.a.

* Elsby R, et al., Eur J Pharm Sci. 2011, 43:41-9

Conclusions

- Corning Life Sciences offers a broad portfolio of ABC transporter vesicle products that feature robust performance with a high signal-to-noise ratio.
- The K_m of selected probe substrates and IC_{50} values of selected transporter modulators were determined using the Corning® Gentest™ ABC Transporter Vesicles and Assay Kit. The data were comparable with literature reports.
- Methods were developed and validated for vesicle uptake assay for radiolabeled or non-radiolabeled chemicals using the rapid filtration system and the LC-MS/MS detection approach.
- Inside-out recombinant membrane vesicles are robust and predictive tools to screen MRP2, BSEP, and BCRP inhibitors that can potentially cause liver toxicity or DDI *in vivo*.

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