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Impact of probe compound in MRP2 vesicular transport assays

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ABSTRACT

MRP2 is an efflux transporter that is expressed mainly in the canalicular membrane of hepatocytes, where it expels polar and ionic compounds into the bile. MRP2 is also present in the apical membrane of enterocytes and epithelial cells of proximal tubules of the kidney. Inhibition of MRP2 transport can lead to the accumulation of metabolites and other MRP2 substrates up to toxic levels in these cells. The transport properties of MRP2 are frequently studied with the vesicular transport assay. The assay identifies compounds that interact with MRP2 by measuring the effect of a compound on the transport of a radioactively labeled or fluorescent probe. We have compared the effect of eight selected test compounds (quercetin, disopyramide, paracetamol, indomethacin, diclofenac, estrone-3-sulfate, budesonide, and thioridazine) on the MRP2-mediated transport of three commonly used probes: 5(6)-carboxy-2,7-dichlorofluorescein, leukotriene C_4 and estradiol-17-β-D-glucuronide ($E_2$17βG). Five of the test compounds had different probe-dependent effects on the MRP2-mediated transport, suggesting differences in the transport mechanism of the probes. Our results underline the complexity of substrate recognition by these efflux transporters and the difficulties in directly comparing results obtained with different assays, especially when different probes are used.

1. Introduction

The ATP-binding cassette (ABC) transporters are a family of membrane proteins that are involved in the ATP-dependent efflux of a wide variety of endogenous compounds and drugs from the cells (Schinkel and Jonker, 2003). The ability to promote resistance to chemotherapy has previously been one of the main reasons for interest in the ABC transporters, but recently they have also been recognized to play a role in drug–drug interactions (reviewed in Marquez and Van Bambeke, 2011).

Multidrug resistance associated protein 2 (MRP2, ABCC2) is a member of the ABCC family, which consists of 13 transporters in human. MRP2 is expressed mainly in the canalicular membrane of hepatocytes, where its physiological role is to expel polar and ionic compounds into the bile. Inhibition of MRP2 transport can lead to an accumulation of metabolites and other MRP2 substrates within the cell and result in hepatotoxicity. MRP2 is also present in the apical membrane of enterocytes and epithelial cells of proximal tubules of the kidney. In addition, enhanced expression of MRP2 has been observed in various cancer cells, but its involvement in multidrug resistance during cancer therapy is currently unclear (reviewed in Jemnitz et al., 2010). The substrates of MRP2 include endogenous glucuronide, sulfate, and glutathione conjugates, such as leukotriene C_4 (LTC_4) andestradiol-17-β-D-glucuronide ($E_2$17βG), as well as different polar and anionic drugs and drug conjugates (reviewed in Jemnitz et al., 2010).

Many of the MRP2 substrates have low membrane permeability, which makes them difficult to study in cell-based assays. The vesicular transport assay with inverted membrane vesicles enables direct interaction of the MRP2 transporter with the compounds added to the reaction buffer. Therefore, it is a widely used *in vitro* method to identify substrates, inhibitors, or modulators of MRP2 (Bodo et al., 2003; Chu et al., 2004; Heredi-Szabo et al., 2008, 2009; Jemnitz et al., 2010; Pedersen et al., 2008; Zelcer et al., 2003). It is possible to directly measure MRP2 mediated transport of a radioactively labeled or fluorescent compound. However, for unlabeled compounds, particularly in high-throughput screening assays, it is common to indirectly

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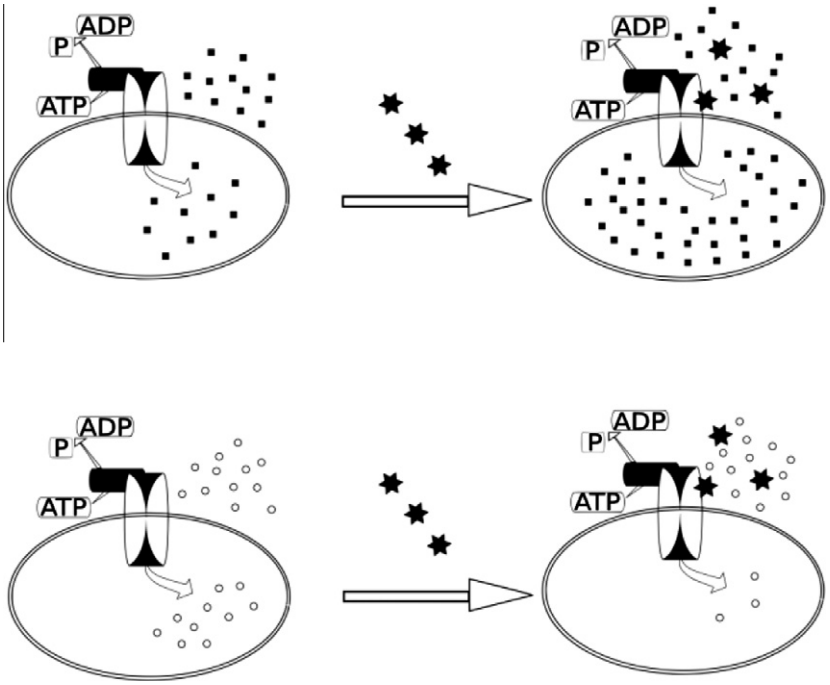

Fig. 1. A schematic explaining the vesicular transport assay. The inverted membrane vesicles enable direct interaction of the MRP2 transporter with compounds added to the reaction buffer. The uptake of the fluorescent or radiolabeled probe (black square or open circle) into the inverted membrane vesicle is ATP dependent. Addition of a test compound (star) can lead to higher accumulation of probe compound in the vesicle (black squares) or decrease the amount of probe compound in the vesicle (open circle). The same compound can act as an inhibitor for one probe and as a stimulator for another probe.

assay the interaction between unlabeled compounds and the transporter proteins by measuring the effect on the transport of probe compounds, such as fluorescent 5(6)-carboxy-2,7-dichlorofluorescein (CDCF) or radio-labeled compounds (Fig. 1). Commercial MRP2 vesicular transport kits with radio-labeled $E_2$17βG and LTC_4, or fluorescent CDCF probes are available and the assay system is frequently used to identify compounds interacting with MRP2. The transport mechanism of these probes by MRP2 vary to some extent, as co-operativity has been reported for $E_2$17βG (Bodo et al., 2003; Zelcer et al., 2003), but not for CDCF or LTC_4 (Heredi-Szabo et al., 2008). $E_2$17βG has been proposed to bind to MRP2 at two distinct binding sites, called the modulator (M) and the substrate (S) sites (Bodo et al., 2003; Zelcer et al., 2003). These two sites would be co-operative; a compound binding to the M site would stimulate the transport of another compound by enhancing the substrate binding affinity at the S site. According to an alternative theory, the compounds would bind to distinct areas within one large binding site, in analogy to the binding cavity observed in the crystal structure of murine P-glycoprotein (ABCB1) (Aller et al., 2009). In contrast to $E_2$17βG, there has been no report of stimulation of LTC_4 transport by MRP2. Heredi-Szabo and co-workers have suggested that both CDCF and LTC_4 are transported according to a one-site model and CDCF could be used as a surrogate for studying LTC4 transport by MRP2 (Heredi-Szabo et al., 2008). Recently, however, verapamil has been shown to stimulate MRP2-mediated CDCF transport (Munic et al., 2011).

Despite the broad use of the three different probe compounds in MRP2 transport studies, the assays have not been compared with each other. Here, we have compared the effects of eight compounds (quercetin, disopyramide, paracetamol, indomethacin, diclofenac, estrone-3-sulfate, budesonide, and thioridazine) on the transport of LTC_4, $E_2$17βG and CDCF in the MRP2 vesicular transport assays. The results revealed that, in addition to similarities, there are intriguing differences between them.

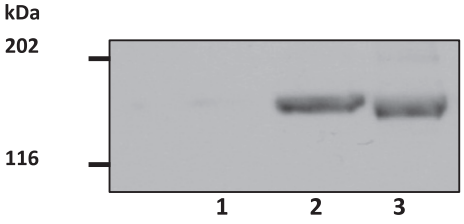

Fig. 2. Immunoblot analysis of human MRP2 expression in membrane vesicles. Fifty micrograms of membrane proteins from Sf9 vesicles were resolved on a 7.5% SDS–polyacrylamide gel and the expression of human MRP2 was verified by immunoblotting with an anti-MRP2 antibody. (1) Non-infected Sf9 vesicles, (2) commercial human MRP2-Sf9 vesicles (Sigma–Aldrich) and (3) the human MRP2-Sf9 vesicles prepared in this study.

2. Material and methods

2.1. Materials

Cloned human MRP2, pGEM3-MRP2, was a kind gift from Dr. Piet Borst (The Netherlands Cancer Institute). HyQ®SFX-Insect MP medium was obtained from Hyclone (Logan, UT, USA). [^{3}H]-$E_2$17βG (1.0 mCi/ml) and [^{3}H]-LTC_4 (0.01 mCi/ml) was purchased from Perkin Elmer (Boston, MA, USA). $E_2$17βG, CDCF, LTC_4, budesonide, diclofenac, indomethacin and estrone-3-sulfate, benzbromarone and ATP were obtained from Sigma–Aldrich (St. Louis, MO, USA). Disopyramide, thioridazine and timolol were purchased from MP biomedicals (Solon, OH, USA). Quercetin was obtained from Merck (Darmstadt, Germany) and paracetamol from Oriola (Espoo, Finland). MRP2-expressing Sf9-vesicles were obtained from Sigma–Aldrich for control experiments.

2.2. Preparation of MRP2 membrane vesicles

The cDNA of human *MRP2* (*ABCC2*) was subcloned from pGEM3-MRP2 into the *Bam*HI/*Xho*I-sites of pFastBacI shuttle vector for