# Use of a Genetically Modified Cell Culture Model to Investigate Human Breast Cancer Resistance Protein-mediated Milk Secretion of Drugs

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**Abstract**

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Over 90% of breastfeeding women use at least one medication, which can expose their infants to drugs and potential toxicity. The Breast Cancer Resistance Protein (BCRP) transporter, encoded by the *ABCG2* gene, is extensively studied in lactation. BCRP protein expression is elevated in lactating mammary epithelial cells (MECs). It is localized on the apical membrane of the MEC, where it plays a pivotal role in the secretion of endogenous and exogenous compounds into breast milk. BCRP actively transports nutrients such as riboflavin into milk, supporting infant development. However, BCRP can actively transport drugs and other xenobiotics into breast milk, increasing the risk of infant drug exposure and toxicity. Currently, there is no validated in vitro human mammary epithelial cell (hMEC) model to assess BCRP-mediated drug secretion during lactation. The goal of this study is to validate the MDCK-hBCRP$^{cMDR1KO}$ cell line as an in vitro model for evaluating BCRP-mediated drug transport into breast milk under

physiologically relevant pH conditions. The MDCK-hBCRP$^{cMDR1KO}$ cell line that was engineered to overexpress human BCRP while lacking endogenous canine P-glycoprotein (P-gp), allows us to isolate BCRP-mediated transport without confounding effects from canine P-gp, which shares overlapping substrate specificity with BCRP. We conducted bidirectional transport assays with cimetidine at apical pH 7.0 (human breast milk), pH 6.5 (intestine), and pH 7.4 (plasma). There was an approximately 2-fold increase in the efflux ratio compared to the MDCK$^{cMDR1KO}$ (control) cells, and the B-to-A transport of cimetidine was inhibited by the BCRP inhibitor KO143. Cimetidine transport was comparable across the three pHs. Our data suggest that, in contrast to membrane vesicles that showed increased BCRP activity under acidic conditions, pH had little impact on cimetidine transport in the monolayer model. These findings highlight that the MDCK-hBCRP$^{cMDR1KO}$ in vitro system is a useful system to study BCRP-mediated drug transport and should be further investigated as a versatile platform to evaluate BCRP-mediated drug secretion and drug-nutrient interaction at the blood-milk barrier.

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# Introduction

Breastfeeding is recognized as a vital nutritional source for infants, providing essential nutrients and immune protection during the critical early months of life (Beers et al., 2025; Camacho-Morales et al., 2021). However, the use of medication during lactation can be a complex clinical challenge, as many lactating women take prescription or over-the-counter medications while breastfeeding, potentially exposing their infants to unintended drug exposure through breast milk, where this exposure can lead to adverse effects, such as infant toxicity, including atenolol-associated bradycardia and cyanosis (Beers et al., 2025; Eyal et al., 2010; Schimmel et al., 1989).

The assessment of drug safety during lactation is significantly hindered by ethical constraints, where it is impossible to conduct pharmacokinetic studies on every drug the mother takes and obtain lactating human breast biopsies (Subramani et al., 2024; Wang et al., 2017). Therefore, to study drug secretion into breast milk, predictive models have been developed to estimate milk-to-plasma (M/P) ratios based on the drug's physicochemical properties, milk pH, free fractions in serum/plasma and milk, and principles of passive diffusion (Begg et al., 1992; Begg & Atkinson, 1993; Maeshima et al., 2023; Oo et al., 1995; Zhang et al., 2022). However, these approaches do not account for active transport processes, which can significantly underestimate the concentrations of actively secreted drugs in breast milk (Zhang et al., 2022). For instance, Oo et al. characterized cimetidine transfer into human milk. They found that the observed milk/serum (M/S) ratio was around 5 times higher than the M/S ratio predicted by passive diffusion (Beers et al., 2025; Oo et al., 1995). This indicates the significant role active

transport processes may play in the drug transfer into human milk, which may affect infant drug exposure.

Among the transporters expressed in the mammary gland, Breast Cancer Resistance Protein (BCRP, also known as ABCG2) has been found to play a major role in the secretion of drugs into breast milk (Alcorn et al., 2002; Mao & Unadkat, 2014; Sychterz et al., 2024). BCRP is a member of the ATP-binding cassette (ABC) transporter family, and several studies have shown that BCRP's mRNA expression is significantly upregulated in mammary epithelial cells (MECs) during lactation compared to non-lactating MECs (Alcorn et al., 2002; Beers et al., 2025; Sychterz et al., 2024). In addition, in Bcrp knockout mice, the M/P ratios of drugs like cimetidine were substantially reduced, which supports BCRP's role in drug secretion into breast milk (Jonker et al., 2005). In addition, BCRP transports a wide range of substrates, including therapeutic drugs such as cimetidine, topotecan, and acyclovir, as well as endogenous compounds like riboflavin (vitamin B2), an essential nutrient for infant development (Beers et al., 2025; Mao & Unadkat, 2014; van Herwaarden et al., 2006). This indicates BCRP's dual role in the disposition of xenobiotics and nutrients, raising the concern of potential drug-nutrient interaction where medication use could modify nutrient composition in the milk.

Previous studies using membrane vesicles and cells have demonstrated that BCRP transports substrates more efficiently under acidic conditions. For example, methotrexate transport by membrane vesicles overexpressing BCRP is 5-fold higher at pH 5.5 compared to physiological pH, and cells overexpressing BCRP exhibit 3-fold higher resistance to mitoxantrone at pH 6.5 compared to physiological pH (Breedveld et al., 2007). This increase in transport could be due to changes in the ionization state of both BCRP and its substrates. Given the fluctuating pH 6.6 to 7.4 environment in breast milk throughout lactation, this may

significantly impact BCRP activity in MEC cells, potentially impacting drug exposure in nursing infants (Beers et al., 2025; Gan et al., 2019; Jen Filatava et al., 2023; Morriss et al., 1986).

Despite these significant findings, a validated in vitro human mammary epithelial cell model for studying BCRP activity in lactation conditions has yet to be developed. Available MEC models, including immortalized and primary human MECs, face several limitations. These include the inconsistent expression levels of transporters, challenges in maintaining and achieving proper epithelial barrier functions, and the lack of robust expression of key transporters, limiting their applicability for mechanistic transport studies (La Mantia et al., 2024).

Among these models, Madin-Darby Canine Kidney (MDCK) cells expressing a human drug transporter are commonly used for drug transport studies in vitro. MDCK cells overexpressing an efflux drug transporter, such as P-glycoprotein (P-gp) or BCRP, are routinely used in the assessment of drug transport and drug interactions in the intestine and the blood-brain barrier (C L Lam & Rajaraman, 2012). P-gp (also known as MDR1 or ABCB1) is one of the major ABC efflux transporters that play a critical role in drug disposition alongside BCRP by actively pumping diverse endogenous and exogenous compounds (Sharom, 2011). P-gp exhibits significant substrate overlap with BCRP, with both transporters sharing numerous common substrates, including topotecan, imatinib, and various tyrosine kinase inhibitors (Agarwal et al., 2011). However, wild-type MDCK cells endogenously express canine P-gp (cP-gp), which exhibits substrate overlap with BCRP (C L Lam & Rajaraman, 2012). While Ito et al. previously used BCRP-expressing MDCK cells to evaluate drug transfer into milk, their study was limited by the presence of endogenous canine P-gp (cP-gp), which can transport many of the same substrates as BCRP, making it difficult to attribute the observed transport activity exclusively to BCRP (Ito et al., 2015). Recently, the generation of the $MDCK^{cMDR1KO}$ using CRISPR-Cas9

gene-editing technology was reported, in which the endogenous cP-gp was knocked out (Simoff et al., 2016). This genetic modification eliminates the contribution of cP-gp, allowing for more specific assessment of other transporters (Simoff et al., 2016). To distinguish BCRP-mediated transport, the MDCK-hBCRP$^{cMDR1KO}$ was generated, where the MDCK$^{cMDR1KO}$ was stably transfected to express human BCRP while lacking endogenous canine P-glycoprotein (P-gp), thereby eliminating potential substrate overlap with cP-gp and providing a clean system for investigating BCRP activity (Wegler et al., 2021).

The goal of this thesis research was to explore the use of the MDCK-hBCRP$^{cMDR1KO}$ cell line as an in vitro model system to evaluate the BCRP-mediated drug transport into milk. Our experimental design specifically focused on the mammary gland environment by conducting transport studies at pH 7.0 on the apical side. Additionally, we evaluated BCRP's activity under pH conditions representative of other physiological environments, including pH 6.5 to simulate the acidic intestinal environment and pH 7.4 to represent systemic plasma conditions. By using a cleaner system and integrating physiological pH conditions, this research may provide a foundation for developing more accurate predictive models of BCRP's drug disposition during lactation.

# Materials and Methods

## *Materials*

[$^3$H] Cimetidine (20 Ci/mmol) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). KO143 was purchased from MedChemExpress (Monmouth Junction, NJ, USA). Lucifer Yellow (LY) was purchased from MP Biomedicals (Irvine, CA). Cell culture media and other reagents were purchased from Thermo Fisher Scientific (Rockford, IL, USA). The bicinchoninic acid (BCA) protein assay kit was purchased from Pierce Chemical.

## *Cell Culture*

Madin-Darby canine kidney (MDCK) cells knocked out for endogenous canine P-gp ($MDCK^{cMDR1KO}$) and $MDCK^{cMDR1KO}$ cells transfected with human breast cancer resistance protein ($MDCK\text{-}hBCRP^{cMDR1KO}$) were kindly provided by Dr. Xiaomin Liang from Gilead with the permission of Dr. Per Artursson at the Uppsala University, who generated the $MDCK^{cMDR1KO}$ model using CRISPR-Cas9 gene-editing technology (Simoff et al., 2016). The MDCK cells were seeded with Dulbecco's modified Eagle's medium (high glucose, GlutaMax™ Supplement) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 $\mu$g/ml streptomycin. The cells were then maintained with a selective antibiotic at 1 μg/mL puromycin. Cells were cultured in a 37°C humidified incubator with 5% $CO_2$.

## *Transport Studies in MDCK-hBCRP$^{cMDR1KO}$ and MDCK$^{cMDR1KO}$ Cells*

Cimetidine flux across the MDCK monolayers was determined using a previously described protocol (Yin et al., 2015). Bidirectional transport assays were conducted in $MDCK\text{-}hBCRP^{cMDR1KO}$ and $MDCK^{cMDR1KO}$ cells. Cells were seeded on 12-well Transwell inserts (0.4

μm polyester membrane; Corning) at a density of $4.85 \times 10^5$ cells/cm$^2$ and cultured for 5 days to form confluent monolayers. Transport experiments were performed under pH gradient conditions (basolateral pH 7.4, apical pH 7.0), reflecting the average pH of breast milk from our ongoing clinical study. The integrity of the MDCK monolayer was verified by measuring the transepithelial electrical resistance (TEER) using the Millicell-ERS system (EMD Millipore, Bedford, MA) before each experiment. Only data from monolayers with TEER values of around 180 Ω cm$^2$ were accepted. In addition, proper formation of tight junctions was also verified by measurement of transepithelial flux of lucifer yellow (50 uM). Only data from inserts with less than the lucifer yellow's apparent permeability (Papp) $< 2.0 \times 10^{-6}$ cm/s over the 2-hour time course were accepted. After removing the cell culture media from both sides of the inserts, the cells were carefully washed twice with warm KRH buffer (pH 7.4). For basal-to-apical (B-to-A) transport, 0.5 ml KRH buffer (pH 7.0) was added to the A chamber, and transport was initiated by adding 1.5 ml KRH (pH 7.4) containing cimetidine (10uM) and lucifer yellow (50 uM) to the B chamber. Similarly, for apical-to-basal (A-to-B) transport, 1.5 ml KRH buffer (pH 7.4) was added to the B chamber, and transport was initiated by adding 0.5 ml KRH (pH 7.0) containing cimetidine and lucifer yellow to the A chamber. To measure the 2-hour transcellular transport, 100 μL was collected from the receiving chamber at each time point and replaced with an equal volume of fresh buffer. Fluorescence measurements of lucifer yellow were performed from a top-read position in a Synergy HTX plate reader (BioTek, Winooski, VT) using a 420/50-nm excitation and 528/20-nm emission filter set. The radioactivity of the collected aliquots was determined by a liquid scintillation counter (Tri-Carb B3110TR; PerkinElmer). Experiments were performed using n = 6 Transwell inserts per group. Data were presented as mean ± S.D. of all inserts.

### *Validate the Presence of hBCRP Activity in the MDCK-hBCRP$^{cMDR1KO}$ Cell Line*

The expression of BCRP in the MDCK-hBCRP$^{cMDR1KO}$ cells was validated through cimetidine transport studies in the presence and absence of the selective BCRP inhibitor KO143. In the bidirectional transport assays, KO143 was added to both apical and basolateral chambers at a final concentration of 1 μM (IC50 is around 9.7 nM), which has been shown to completely inhibit BCRP-mediated transport (Allen et al., 2002; Weidner et al., 2015; Wright et al., 2011). Bidirectional transport assays were conducted according to the procedures described above, using KRH buffer (apical pH 7.0, basolateral pH 7.4) and cimetidine at a concentration of 10 μM. Monolayer integrity was verified by measuring TEER and lucifer yellow flux as previously described. Experiments were performed using n = 6 Transwell inserts per group. Data are presented as mean ± S.D. of all inserts.

### *Effect of Apical pH on Cimetidine Transport in MDCK-hBCRP$^{cMDR1KO}$ and MDCK$^{cMDR1KO}$ Cells*

To assess the influence of apical pH on cimetidine transport, bidirectional transwell assays were performed utilizing MDCK-hBCRP$^{cMDR1KO}$ and MDCK$^{cMDR1KO}$ cell lines. The transport studies were conducted under three apical pH conditions (6.5, 7.0, and 7.4), while maintaining the basolateral pH at 7.4, to replicate physiologically relevant environments, such as intestinal conditions (pH 6.5) and the average pH of human breast milk (pH 7.0), as documented in our ongoing clinical studies. Given cimetidine's low logP (0.4-0.7) and the pKa of 6.8 (Avdeef & Berger, 2001; Boom et al., 1994; Jantratid et al., 2006; Song et al., 2017), which indicates limited passive diffusion, the duration of the cimetidine transport assay was extended from 2 to 4

hours to ensure adequate drug movement across the cell monolayers. Samples were collected from both the respective receiver compartments. Monolayer integrity was evaluated, and the radioactivity of the collected aliquots of cimetidine was measured as previously described. Experiments were conducted using n = 6 Transwell inserts per group, and data were presented as mean ± S.D. of all inserts.

*Data Analysis*

Transwell studies were performed in MDCK cells using n = 6 per condition. Data are reported as mean ± S.D. Data were plotted and fitted using linear regression in GraphPad Prism 10 (GraphPad Software Inc., La Jolla, CA). The apparent permeability (Papp) of cimetidine across cell monolayers was calculated using the following equation:

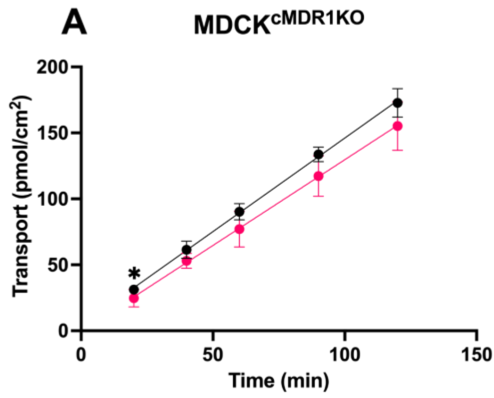
$$P_{app} = \frac{\left(\frac{dQ}{dt}\right)}{A \times C_0}$$

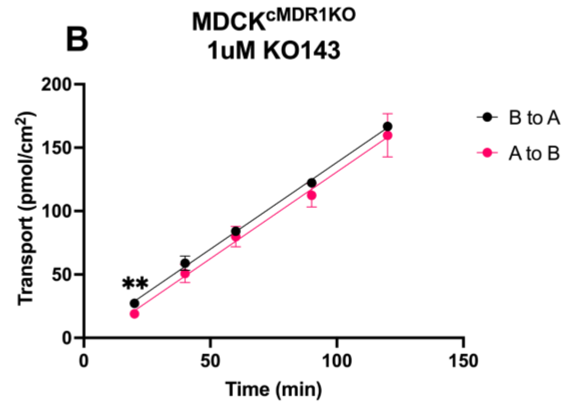
Where Q is the amount of compound transported over time t, A is the insert membrane surface area, and $C_0$ is the initial compound concentration in the donor chamber.

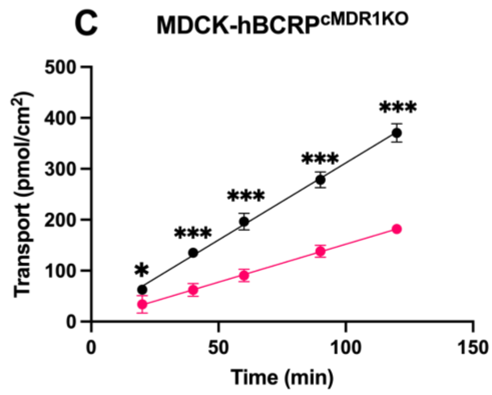
Statistical significance was determined using a two-way ANOVA with Bonferroni's multiple comparison tests. A *P* value less than 0.05 was considered statistically significant. Cimetidine's transport rate was evaluated using multiple unpaired Student's t-test. Efflux ratios were analyzed using either unpaired Student's t-test, one-way ANOVA, or one-way ANOVA with Dunnett's

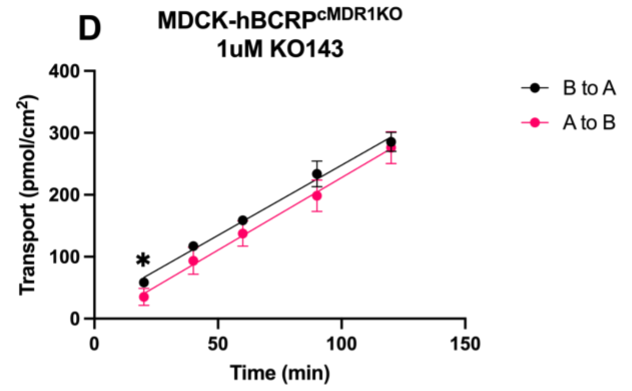
multiple comparison test, as appropriate. A P-value less than 0.05 was considered statistically significant.

# Results



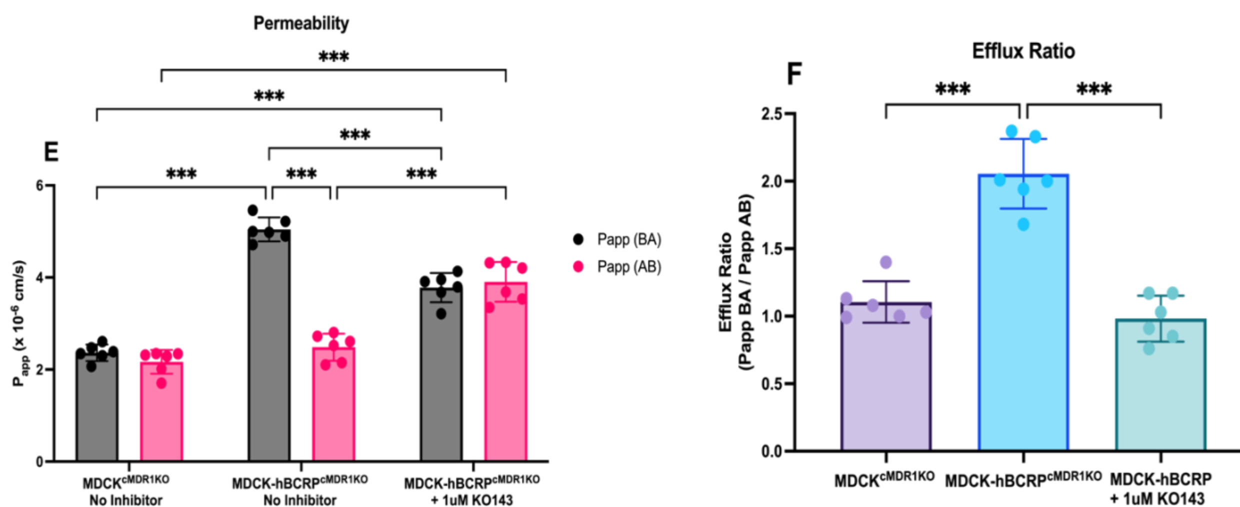
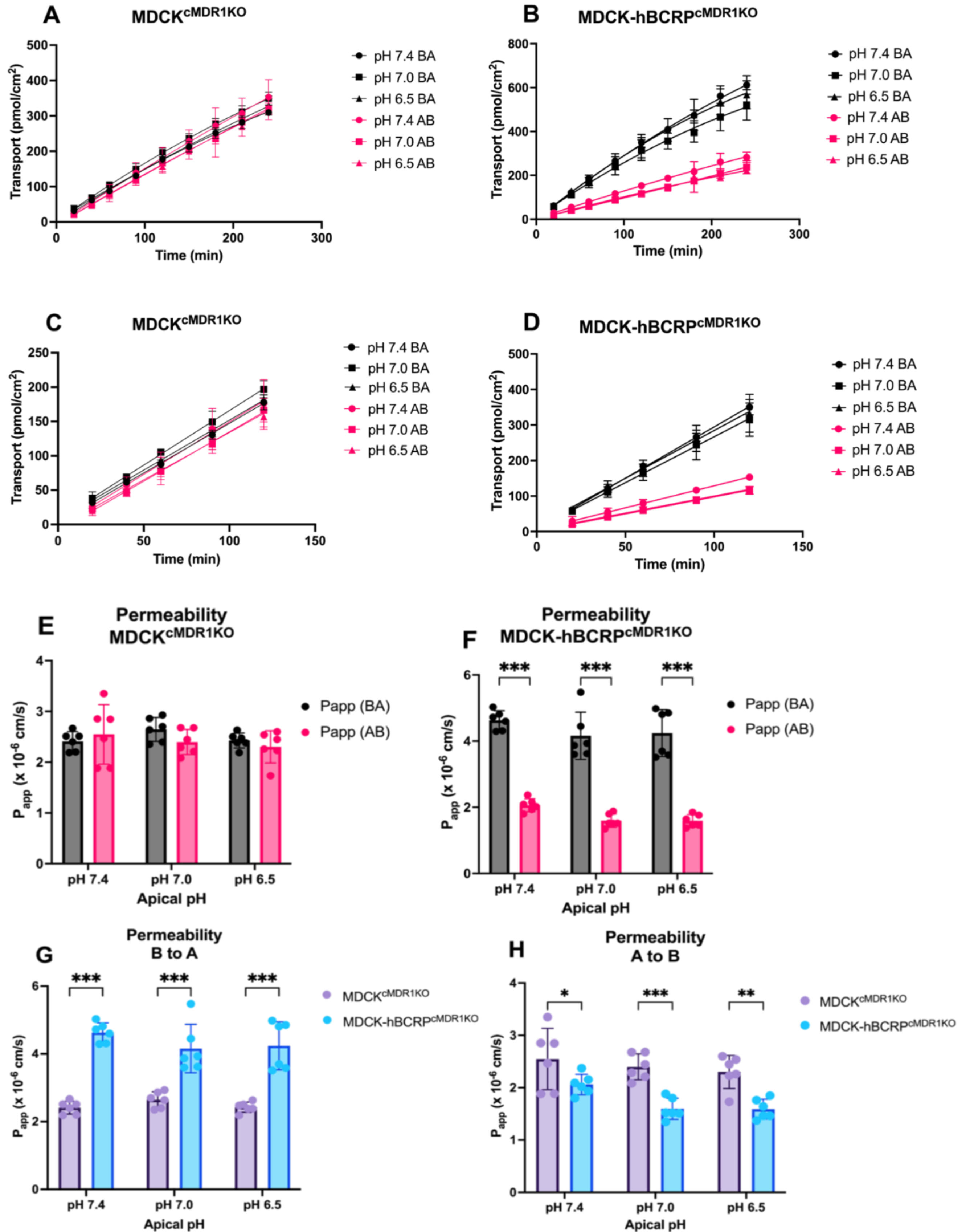



**Figure 1. Transcellular flux, permeability, and efflux ratio of cimetidine in MDCK-hBCRP$^{cMDR1KO}$ and MDCK$^{cMDR1KO}$ (control) cells.** MDCK cells were incubated in KRH transport buffer containing 10 µM cimetidine in either the basolateral (black circle) or apical (pink circle) chamber (A-D) and also in the presence of 1µM KO143 (B and D). A 100 µL aliquot of buffer was taken from the receiving chamber, and the radioactivity of cimetidine was measured. The pH of the basolateral and apical chambers was 7.4 and 7.0, respectively. Permeability of cimetidine was calculated using the equation described in the Materials and Methods section (E). The efflux ratio of cimetidine was calculated (F). Data were fitted with linear regression. Transport and permeability were compared with those in the apical to basolateral direction (A to B) **(*$P$ < 0.05; **$P$< 0.01; ***$P$<0.001)**.

*Functional Validation of MDCK-hBCRP$^{cMDR1KO}$ Cells*

To evaluate the activity of the expressed ATP-binding cassette transporter, BCRP, a time-dependent flux of [$^3$H] cimetidine, was measured in the presence and absence of a known potent BCRP inhibitor, KO143 (1 µM), in both MDCK$^{cMDR1KO}$ (control) and MDCK-hBCRP$^{cMDR1KO}$ cell lines (Fig. 1). Transport studies were conducted under pH gradient conditions, with an apical pH of 7.0 and a basolateral pH of 7.4, reflecting the physiological conditions of the average pH of human breast milk from our ongoing clinical study. As illustrated in Fig. 1C, the transport of

cimetidine in the basolateral-to-apical (B-to-A) direction was significantly greater than in the apical-to-basolateral (A-to-B) direction in the MDCK-hBCRP$^{cMDR1KO}$cells. In the presence of KO143, the B-to-A transport of cimetidine decreased in the MDCK-hBCRP$^{cMDR1KO}$ cells (Fig. 1D). In contrast, in the MDCK$^{cMDR1KO}$ (control) cells, cimetidine transport was slower, non-polarized, and not affected by KO143 (Fig. 1A &B). In both the control and MDCK-hBCRP$^{cMDR1KO}$ cells, the cimetidine flux rate remained constant over the 120-minute time course. Based on this time course, cimetidine permeability was calculated and shown in Fig. 1E. The permeability of cimetidine in the B-to-A direction was around 2-fold higher in MDCK-hBCRP$^{cMDR1KO}$ cells compared to the control cells, and with the presence of KO143, the B-to-A direction in the MDCK-hBCRP$^{cMDR1KO}$ decreased from 5.04 × 10^-6 cm/s to 3.78 × 10^-6 cm/s. The calculated efflux ratios were 1.10 in control cells and 2.03 in MDCK-hBCRP$^{cMDR1KO}$, which was reduced to 0.95 in the presence of KO143 (Fig.1F). Together, these data suggest that BCRP expressed in the MDCK$^{cMDR1KO}$ cells actively transports cimetidine, and KO143 completely inhibits this transport.


A
MDCK^cMDR1KO
Transport (pmol/cm²)
Time (min)
pH 7.4 BA
pH 7.0 BA
pH 6.5 BA
pH 7.4 AB
pH 7.0 AB
pH 6.5 AB
B
MDCK-hBCRP^cMDR1KO
C
MDCK^cMDR1KO
D
MDCK-hBCRP^cMDR1KO
E
Permeability
MDCK^cMDR1KO
Papp (BA)
Papp (AB)
Apical pH
pH 7.4
pH 7.0
pH 6.5
F
Permeability
MDCK-hBCRP^cMDR1KO
G
Permeability
B to A
MDCK^cMDR1KO
MDCK-hBCRP^cMDR1KO
H
Permeability
A to B

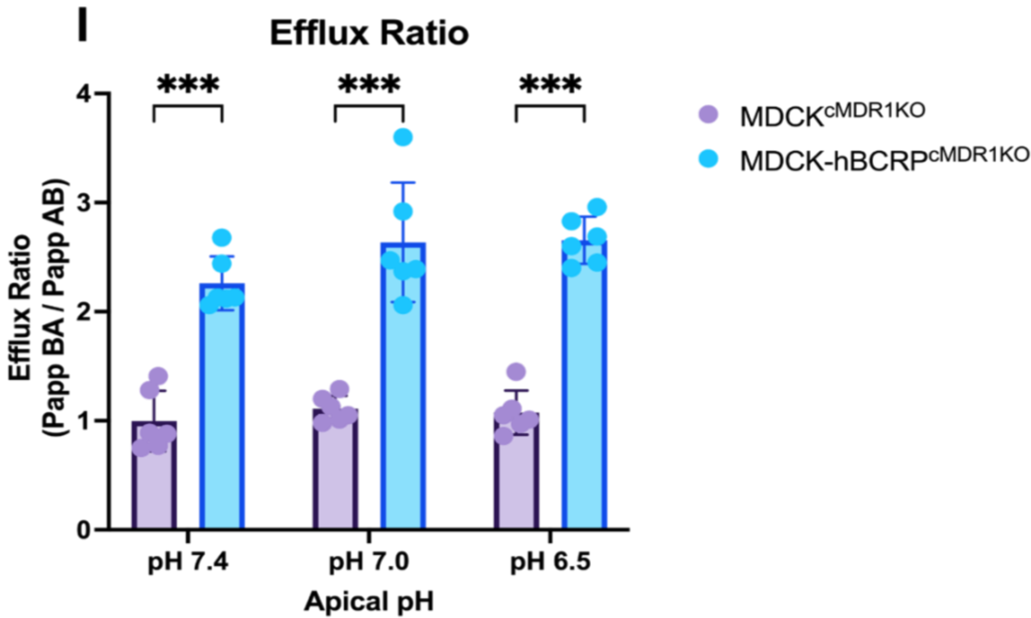



**Fig. 2** Transcellular flux (4 hours and 2 hours), permeability, and efflux ratio of cimetidine in MDCK-hBCRP$^{cMDR1KO}$ and MDCK$^{cMDR1KO}$ (control) cells. MDCK cells were incubated in KRH transport buffer containing 10 µM cimetidine in either the basolateral or apical chamber with pH 7.4, 7.0, and 6.5 for 4 hours (A and B) and 2 hours (C and D). A 100 µL aliquot of buffer was taken from the receiving chamber, and the radioactivity of cimetidine was measured. The permeability of cimetidine was calculated up to 2 hours using the equation described in the Materials and Methods section (E-H). The efflux ratio of cimetidine was calculated (I). Data were fitted with linear and nonlinear regression. Transport and permeability in the basolateral to apical direction (B to A) were compared with that in the apical to basolateral direction (A to B) **(****$P$* **< 0.05; *****$P$***< 0.01; ***$P$<0.001)**.

## *Effect of apical pH on Cimetidine Transport by BCRP*

To assess whether variations of apical pH influence the transport of cimetidine by BCRP, time-dependent bidirectional transport assays were conducted at apical pH levels of 6.5, 7.0, and 7.4 in MDCK$^{cMDR1KO}$ and MDCK-hBCRP$^{cMDR1KO}$ monolayers (Fig. 2). Given cimetidine's low LogP value, approximately 0.4-0.7 (Avdeef & Berger, 2001; Jantratid et al., 2006) and consequently limited membrane permeability, transport rate was conducted over an extended 4-hour period to ensure adequate flux in both cell lines (Fig. 2A and B). During the initial 2-hour

period, both cell lines demonstrated a steady linear increase in cimetidine transport across all apical pH conditions (Fig. 2C & D). However, as transport approached 4 hours, the B-to-A transport rate (as reflected by slope) in the BCRP-expressing cells started to decrease, possibly reflecting a diminished concentration gradient resulting from progressive depletion of cimetidine in the donor compartment. As shown in Fig. 2B and 2D, cimetidine transport in the basolateral-to-apical (B-to-A) direction was significantly greater than in the apical-to-basolateral (A-to-B) direction across all tested pH conditions in the MDCK-hBCRP$^{cMDR1KO}$ cells, consistent with active BCRP-mediated efflux. However, no significant differences in B-to-A transport were observed among the three apical pH conditions, suggesting that pH variation does not substantially affect BCRP-mediated cimetidine efflux in bidirectional studies (Fig. 2B and D). MDCK$^{cMDR1KO}$ cells, which lack BCRP expression, exhibited significantly slower cimetidine transport in B-to-A directions. Apical pH also had no impact on cimetidine transport across the MDCK$^{cMDR1KO}$ monolayer in either direction (Fig. 2A & C).

The apparent permeability coefficient (Papp) and efflux ratio values were calculated using data from the linear transport phase occurring within the 0–2-hour time interval in both cell lines (Fig. 2E-I). The Papp in both cell lines was not affected by apical pH (Fig. 2E and F). Papp in the B-to-A direction was significantly higher in MDCK-hBCRP$^{cMDR1KO}$ cells compared to MDCK$^{cMDR1KO}$ cells across the different apical pHs, confirming active efflux mediated by BCRP (Fig. 2G). On the other side, the Papp in the A-to-B direction was significantly higher in the MDCK$^{cMDR1KO}$ cells than in the MDCK-hBCRP$^{cMDR1KO}$, consistent with apical efflux by BCRP (Fig.2H). Importantly, no significant differences in efflux ratios and B-to-A Papp values were observed across the different apical pH conditions in either cell line (Fig. 2I),

demonstrating that BCRP-mediated cimetidine transport is independent of apical pHs tested in this study.

# Discussion

Despite the well-recognized importance of BCRP in drug secretion into breast milk (Ito et al., 2015; Sychterz et al., 2024; Zhang et al., 2022), there is no valid in vitro model to predict BCRP-mediated drug transport into human breast milk. In this study, we explored the use of a genetically modified cell line (MDCK-hBCRP$^{cMDR1KO}$) as an in vitro model system to evaluate the BCRP-mediated drug transport into milk under physiological pH. Our results demonstrated the functional transport of cimetidine by BCRP in this model and showed that apical pH had little impact on cimetidine transport in the monolayer model.

Cimetidine is actively secreted into human breast milk, with clinical studies demonstrating a milk-to-plasma (M/P) ratio exceeding passive diffusion predictions (Beers et al., 2025; Begg et al., 1992; Oo et al., 1995; Zhang et al., 2022). These differences in the M/P ratios are thought to be due to active transport processes specifically mediated by BCRP, which is known to be highly expressed in human mammary epithelial cells (MECs) during lactation (Ahmadzai et al., 2022; Beers et al., 2025; Jonker et al., 2005). Several studies confirmed the role of BCRP in mediating cimetidine transport (Beers et al., 2025; Ito et al., 2015; Pavek et al., 2005; Sychterz et al., 2024; Zhang et al., 2022). Previously, Ito et al. conducted a transwell study using conventional MDCKII cells expressing human BCRP and observed an efflux ratio of 3.27 for cimetidine (Ito et al., 2015). However, their control MDCK cells also exhibited polarized cimetidine transport with the baseline efflux ratio of 2.00. These data suggest the presence of endogenous transporters, potentially endogenous canine P-glycoprotein (cP-gp), which also contribute to cimetidine transport in their BCRP transfected cells, making it hard to dissect the specific contribution of BCRP in cimetidine transport. In contrast, our study utilized an engineered cell line where cP-gp is knocked out using CRISPR technology. Interestingly, in

our cP-gp knockout control (MDCK$^{cMDR1KO}$) cells, cimetidine showed an efflux ratio close to unity (Fig. 1A), demonstrating no transporter-mediated directional cimetidine flux. These data strongly suggest that cP-gp is responsible for the polarized cimetidine transport in the control cells in the Ito study (Ito et al., 2015). In our MDCK-hBCRP$^{cMDR1KO}$ cells, we observed cimetidine efflux ratio of ~ 2 which was reduced to 1.0 in the presence of KO143 (Fig. 1D). Together, these data suggest that the MDCK-hBCRP$^{cMDR1KO}$ cells is a cleaner system for assessing BCRP-mediated transport, especially for compounds that are dual substrate of BCRP and P-gp.

Despite recent advances in understanding BCRP-mediated transport in the lactating mammary gland, significant challenges remain in predicting clinical outcomes. Through systematic literature compilation, Ito et al. established a representative clinical M/P ratio of 4.18 for cimetidine, which they incorporated into their mechanistic transport modeling framework. However, even after incorporating quantitative BCRP-mediated transport parameters derived from their bidirectional transport assays, their predictive model still underestimated the observed clinical transport by 3.13-fold, indicating that additional transport mechanisms beyond BCRP may contribute to cimetidine's extensive milk secretion. Hence, there is a need to evaluate the specific contribution of BCRP in milk transfer of cimetidine.

Previous studies have suggested that cimetidine is also a substrate of P-glycoprotein (P-gp) transporter, which is also expressed in human MECs (Karyekar et al., 2003; Taur & Rodriguez-Proteau, 2008, Lee & Wang, unpublished data). However, this dual substrate nature presents methodological challenges for isolating BCRP-specific transport contributions, as both BCRP and P-gp can mediate drug efflux and potentially confound mechanistic interpretations. To address this limitation, we employed the MDCK-hBCRP$^{cMDR1KO}$ cell line as our in vitro

model system, where the MDCK$^{cMDR1KO}$ was genetically modified through the CRISPR-Cas9-mediated genetic knockout of endogenous cP-gp to eliminate substrate overlap and enable precise evaluation of BCRP-specific transport activity (Simoff et al., 2016; Wegler et al., 2021). This system has significant advantages over conventional MDCK-hBCRP models, where MDCK cells are found to express functional endogenous P-glycoprotein activity that can confound interpretation of BCRP-mediated transport mechanisms (Kuteykin-Teplyakov et al., 2010).

Interestingly, the variations in apical pH (pH 7.4, 7.0, 6.5) did not significantly affect BCRP-mediated cimetidine transport (Fig. 2). This finding contrasts with previous studies using membrane vesicles that demonstrated significant pH-dependent BCRP activity, with methotrexate showing a 5-fold increase at acidic pH compared to physiological pH (Breedveld et al., 2007; Li et al., 2011). The reason for this discrepancy is unclear but could be attributed to fundamental differences in experimental systems. Membrane vesicles provide direct exposure of the transporter to substrates and buffer conditions, where BCRP is oriented inside-out with ATP-binding sites and pH-sensitive amino acid residues (such as arginine 482) facing outward. This configuration may allow pH-mediated ionization of the residues that may affect substrate binding and BCRP transport activity. In contrast, cell monolayers reflect the physiological structure of epithelial membranes with distinct basolateral and apical compartments, where substrates must cross cellular barriers before encountering BCRP. Polarized cells possess an endogenous intracellular composition that may regulate the effects of extracellular pH changes, unlike the direct buffer exposure in vesicle preparations. The pH range examined in this study was chosen to reflect physiologically relevant conditions during human lactation. Human breast milk pH typically ranges from 6.6 to 7.4 (Beers et al., 2025; Gan et al., 2019; Jen Filatava et al., 2023), with slight variations occurring throughout lactation. The consistent BCRP activity across this

pH range (Fig. 2) suggests that fluctuations in human breast milk pH during lactation may not significantly impact BCRP-mediated drug transport.

Despite controlling endogenous cimetidine transport and simulating physiological pH, the efflux ratio determined in our study was notably lower than the reported in vivo milk-to-plasma (M/P) ratio of 4.18-5.77 observed in human lactation studies (Ito et al., 2015; Oo et al., 1995) This discrepancy suggests that our in vitro model, while successfully demonstrating specific BCRP functionality, still needs further refinement for predicting in vivo M/P ratio. Several factors can be considered to bridge the gap. First, an important consideration is the differences of the BCRP protein abundance between our MDCK cell model and the lactating human MECs. Future quantification of BCRP protein levels in the MDCK cell line and the human MECs would allow the incorporation of the relative expression factor (REF) to bridge the gap between our in vitro efflux ratios and the higher M/P ratios observed clinically. Second, the in vivo cimetidine transport likely involves cooperative mechanisms between multiple transporters. Studies have demonstrated that cimetidine transfer into milk requires not only BCRP but also organic cation transporter 1 (OCT1) for basolateral uptake (Beers et al., 2025; García-Lino et al., 2019), creating a vectorial transport system that our single-transporter model did not replicate. Additionally, while P-glycoprotein (P-gp/MDR1) expression is significantly downregulated in human MECs during lactation, with studies showing up to a 4-fold decrease in P-gp mRNA expression compared to non-lactating MECs (Ahmadzai et al., 2022; Alcorn et al., 2002; Beers et al., 2025), this reduced P-gp activity may still contribute to enhanced net drug accumulation into human breast milk.  Lastly, human breast milk contains lipids and protein compositions, including milk fat globules, lactoferrin, and α-lactalbumin (Atkinson & Begg, 1988; Beers et al., 2025; Thum et al., 2022), which may enhance drug partitioning that is absent in our standard

culture medium. To improve the cell model, future studies could incorporate human milk or milk-like medium in the apical chamber to mimic the lactating state and transfect the cell model with P-gp and with relevant uptake transporters such as OCT1.

In summary, we validated the MDCK-hBCRP$^{cMDR1KO}$ cell line as an in vitro model for assessing BCRP-mediated drug transport under physiologically relevant pH conditions in lactation. We validated the activity of BCRP in the MDCK-hBCRP$^{cMDR1KO}$ model and observed the pH-independent transport of cimetidine by BCRP. By eliminating endogenous cP-gp, this clean model better reflects BCRP transport of cimetidine. To improve physiological relevance, future enhancements could include adding human milk or milk-like medium apically, co-expressing basolateral uptake transporters, incorporating expression of other efflux transporters like P-gp, and measuring expression of transporters in the MDCK cells and MECs. These improvements could address the critical need for a validated in vitro human MEC model to study BCRP's role in nutrient and xenobiotic secretion during lactation and evaluate drug–drug and drug–nutrient interactions at the blood-milk barrier.

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