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## Short Communication

# Mrp3 Transports Clopidogrel Acyl Glucuronide from the Hepatocytes into Blood

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## ABSTRACT

**Clopidogrel acyl glucuronide (CLP-G) is a major phase II metabolite of clopidogrel generated in the liver for further excretion into urine; however, it is unclear whether CLP-G transports from hepatocytes into blood. Because multidrug resistance-associated protein 3 (MRP3) is predominantly expressed in the sinusoidal side of hepatocytes and preferentially transports glucuronide conjugates of drug metabolites from hepatocytes into bloodstream, we hypothesized that MRP3 could be such an efflux transporter for CLP-G. In this study, we compared the liver-to-plasma ratios of clopidogrel and its metabolites (including CLP-G) between *Abcc3* (ATP-binding cassette, subfamily C, member 3) knockout (KO) and wild-type (WT) mice. We also evaluated the ATP-dependent uptake of clopidogrel and CLP-G as well as estradiol-17$\beta$-D-glucuronide into human recombinant MRP3 inside-out membrane vesicles in the presence or absence of ATP. The results indicated that the liver-to-plasma ratio of CLP-G was 11-fold higher in KO mice than in WT mice, and that uptake of CLP-G (1 or 10 $\mu$M each) into the membrane vesicles was 11.8- and 3.8-fold higher in the presence of ATP than in the presence of AMP, respectively. We conclude that Mrp3 transports CLP-G from the hepatocytes into blood in an ATP-dependent manner.**


## Introduction

Clopidogrel has been selected as an essential medicine for patient care by the World Health Organization (Patel et al., 2015). Despite its widespread use in clinical settings as an antiplatelet drug (Xie et al., 2011; Saeed et al., 2017), clopidogrel continues to draw attention worldwide. Previous studies demonstrated that clopidogrel undergoes extensive metabolism in the liver (Kazui et al., 2010; Xie et al., 2011; Zhu et al., 2013; Savu et al., 2016; Tai et al., 2016). In the human body, ~85% of ingested clopidogrel is rapidly hydrolyzed to clopidogrel carboxylate (CLP-C, an inactive carboxylic acid form), an immediate metabolite, by hepatic carboxylesterase 1 (Zhu et al., 2013); the remaining 15% is metabolized to clopidogrel active metabolite (CAM) by multiple cytochrome P450–mediated two-step oxidative pathways in the liver (Savi et al., 1992, 2000; Kazui et al., 2010; Xie et al., 2011, 2017). Furthermore, the formation of clopidogrel acyl glucuronide (CLP-G) from CLP-C catalyzed by uridine diphosphate-glucuronosyltransferases (UGTs) in the liver is the major elimination pathway of clopidogrel in humans (Silvestro et al., 2011; Tornio et al., 2014), with the mean maximum plasma concentration of CLP-G exceeding 1000 times that of clopidogrel in patients taking this drug. If the carboxylesterase 1–catalyzed hydrolysis (i.e., inactivation) and elimination (e.g., glucuronidation) of clopidogrel were suppressed or severely impaired, its residual fraction would be increased and diverted for its bioactivation to generate more CAM molecules, leading to an enhanced antiplatelet effect (Xie et al., 2011).

Although clopidogrel is considered as a substrate drug of P-glycoprotein (Taubert et al., 2004), little is known about the transporting profile of clopidogrel and its metabolites in the body. Multidrug resistance-associated protein 3 (also known as MRP3, encoded by the gene *ABCC3,* ATP-binding cassette, subfamily C, member 3) functions as an efflux transporter that mediates the export of its substrates from the enterocytes to the hepatic portal system, and from the hepatocytes to the general circulation for systemic exposure. A pilot clinical research study indicated that patients with low *ABCC3* mRNA expression would respond well to clopidogrel and vice versa (Luchessi et al., 2012). Consistent with this finding, we observed that the *Abcc3* knockout (KO) mice exhibit an enhanced platelet response to clopidogrel due to increased CAM formation when compared with wild-type (WT) mice (Tai et al., 2016).

In terms of the fact that Mrp3 is predominantly expressed in the sinusoidal membrane of hepatocytes (Kool et al., 1999; Scheffer et al., 2002; Zelcer et al., 2006; Kitamura et al., 2008) and that MRP3 preferentially transports glucuronide conjugates of drug metabolites from the hepatocytes into bloodstream (Manautou et al., 2005; Zelcer et al., 2005, 2006; Smith and Dalvie, 2012), we hypothesized that CLP-G could be transported from hepatocytes to the blood via MRP3.

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**ABBREVIATIONS:** Abcc3, ATP-binding cassette, subfamily C, member 3; CAM, clopidogrel active metabolite; CAMD, clopidogrel active metabolite derivative; CLP, clopidogrel; CLP-C, clopidogrel carboxylate; CLP-G, clopidogrel acyl glucuronide; $E_217\beta G$, estradiol-17$\beta$-D-glucuronide; IS, internal standard; KO, knockout; LC-MS/MS, liquid chromatography with tandem mass spectrometry; MPB, 2-bromo-3′-methoxyacetophenone; MRP, multidrug resistance-associated protein; WT, wild type.

To test this hypothesis, we used *Abcc3* KO mice to determine whether there could be significantly higher liver-to-blood ratios of CLP-G in KO mice than in WT mice. Furthermore, we used an inverted membrane vesicular transport assay to directly evaluate the ATP-dependent uptake of clopidogrel as well as CLP-C and CLP-G into human recombinant MRP3 "inside-out" membrane vesicles in the presence of ATP versus AMP.

## Materials and Methods

**Animals.** The *Abcc3* KO mice were generated and validated first by the Netherlands Cancer Institute, the Netherlands (Zelcer et al., 2006) and were generously provided for this study. WT mice of FVB strain were purchased from Vital River Laboratories, Beijing, People's Republic of China. All animals were housed in an air-conditioned room with a 12-hour light/dark cycle and had free access to food and water, but were fasted for 12 hours before the studies were performed. All the studies were approved by the Experimental Animal Welfare and Ethics Committee of Nanjing Medical University and were conducted in compliance with the Guidelines for Animal Experimentation, Nanjing Medical University, People's Republic of China.

**Chemicals and Reagents.** Clopidogrel (CLP) bisulfate, piroxicam (internal standard, or IS), and 2-bromo-3′-methoxyacetophenone (MPB) were purchased from Sigma-Aldrich (St. Louis, MO). Racemic CAM derivatized with MPB (i.e., CAMD, or CAM equivalent) and clopidogrel acyl-$\beta$-D-glucuronide (CLP-G) were synthesized by Toronto Research Chemicals (Toronto, Ontario, Canada). Clopidogrel carboxylic acid or carboxylate (CLP-C) was purchased from Santa Cruz Biotechnology (Dallas, TX). Recombinant human MRP3 inside-out membrane vesicles GM0021 (GenoMembrane, Yokohama, Japan), adenosine monophosphate (AMP), adenosine triphosphate (ATP), and estradiol-17$\beta$-D-glucuronide ($E_2 17\beta G$) were purchased from Solvo Biotechnology (Szeged, Hungary). High-pressure liquid chromatography–grade acetonitrile was obtained from Merck (Darmstadt, Germany). Formic acid and other chemicals and solvents used were of analytical grade or above. Deionized water was purified using a Milli-Q system (Millipore, Milford, MA).

**Quantitative Analysis of Clopidogrel and Its Metabolites in Mice.** Male WT and KO mice (aged 6−8 weeks each) were treated with clopidogrel by lavage administration at a single dose of 10 mg/kg, respectively. Blood samples (100 $\mu$l each) were withdrawn from the orbital venous plexus into heparinized polythene tubes pretreated with 2 $\mu$l of 500 mM MPB in acetonitrile at 10 minutes after clopidogrel administration, respectively, and were mixed immediately for the rapid formation of CAMD to keep CAM stable in plasma. Ultimately, each blood sample was separated by centrifugation at 4000 rpm for 10 minutes and kept frozen at −80°C until analysis. Immediately after collecting blood samples, we collected liver specimens by sacrificing mice. The liver tissue was weighed and homogenized (20%, w/v) in normal saline solution containing 50 $\mu$l of 500 mM MPB in acetonitrile. Each sample was separated by centrifugation at 4000 rpm for 10 minutes and kept frozen at −80°C until analysis.

Frozen samples were thawed on ice before homogenization by vortex-mixing. Aliquots (10 $\mu$l) of plasma or liver tissue homogenates, spiked with 10 $\mu$l of piroxicam (IS) working solution (250 ng/ml), were vortex-mixed for 30 seconds. Protein precipitation was then performed by adding 300 $\mu$l of ice-cold acetonitrile by vortex-mixing for 3 minutes. Samples were centrifuged at 14,800 rpm for 20 minutes before the supernatant was transferred to a glass vial. Aliquots of 5 $\mu$l were injected into the liquid chromatography with tandem mass spectrometry (LC-MS/MS) system for quantitative analysis.

The concentrations of clopidogrel, CAM, CLP-C, and CLP-G in plasma and liver tissue as well as piroxicam (IS) were determined by the validated LC-MS/MS method as described elsewhere (Tai et al., 2016; Yin et al., 2016).

**Determination of $E_2 17\beta G$, Clopidogrel, CLP-C, and CLP-G in the Membrane Vesicles.** As a well-characterized substrate for MRP3, $E_2 17\beta G$ was used as a positive control. ATP-dependent uptake of $E_2 17\beta G$ into human recombinant MRP3 membrane vesicles was measured using the LC-MS/MS technology. In brief, human MRP3 vesicle suspensions were loaded onto 96-well flat-bottom tissue culture plates, followed by the addition of $E_2 17\beta G$ (1 $\mu$M), CLP (1 and 10 $\mu$M each), CLP-C (1 and 10 $\mu$M each), or CLP-G (1 and 10 $\mu$M each), respectively. Plates were preincubated for 5 minutes at 37°C. The reactions were started by the addition of 25 $\mu$l of assay buffer (Solvo kit) with ATP or AMP, allowed to proceed for 5 minutes at 37°C, and terminated with 200 $\mu$l of ice-cold "washing mix" (Solvo kit). The solution was transferred to a glass fiber (Type B) filter plate (Millipore, Billerica, MA) and washed 5 times with "washing mix" using a Millipore Multiscreen rapid filtration vacuum manifold. Vesicles were solubilized in acetonitrile:water (80:20, v/v) at room temperature and vacuum collected. Piroxican (20 ng/ml in acetonitrile) was added to all testing samples as the internal standard for LC-MS/MS analysis.

The concentration of $E_2 17\beta G$, CLP, CLP-C, or CLP-G in each well was measured in the presence of ATP or AMP, in which ATP-dependent uptake represents active transport. The uptake ratio is defined as the ratio of uptake amount in the presence of ATP to that in the presence of AMP. For $E_2 17\beta G$, its uptake ratio of greater than 2 demonstrates that the testing system works perfectly, and that the data obtained by that system are reliable and reproducible. Similarly, when the uptake ratio is $\geq$2, the chemical tested is considered as a substrate of human MRP3. All experiments were performed in triplicate, and data presented are expressed as the mean ± S.D. of multiple experiments.

**Statistical Analysis.** All data are expressed as mean ± S.D. Student's two-tailed, unpaired *t* test was used for group comparisons of a single variable. $P <$ 0.05 was considered statistically significant.

## Results and Discussion

**CLP-G Is Identified as an Mrp3 Substrate In Vivo and In Vitro.** Hepatic Mrp3 functions as an efflux transporter that extrudes its substrates from the hepatocytes into bloodstream, so we used the liver-to-plasma ratio of CLP-G to directly reflect differences in CLP-G distribution in the hepatocytes versus blood in the presence or absence of Mrp3 in mice and to further reveal whether CLP-G is a substrate of Mrp3. As shown in Fig. 1A, the liver-to-plasma ratio of CLP-G was 11-fold higher in *Abcc*3 KO mice than in WT mice, and these results strongly suggested that CLP-G is an Mrp3 substrate as anticipated. Furthermore, the liver ratio of CLP-G to CLP-C was 1.8-fold higher in KO mice than in WT mice, but this ratio in blood of KO mice was just 5% of that of WT mice (Fig. 1B), also suggesting that CLP-G may be a substrate of Mrp3.

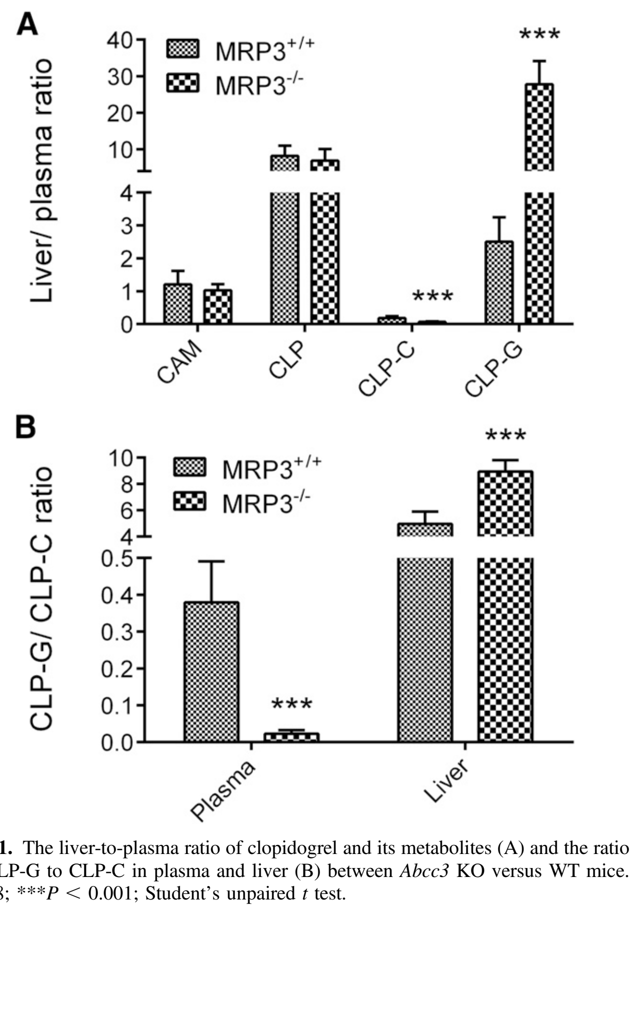


**Fig. 1.** The liver-to-plasma ratio of clopidogrel and its metabolites (A) and the ratio of CLP-G to CLP-C in plasma and liver (B) between *Abcc3* KO versus WT mice. $n$ = 8; ***$P <$ 0.001; Student's unpaired *t* test.

Inverted membrane vesicles have been used primarily to study efflux transporter activity, in particular for ABC transporters. A major advantage of this methodology is that drugs or their metabolites are directly measured with the influx or uptake for substrate or inhibitor interactions with the target transporters (Giacomini et al., 2010).

In this study, "inside-out" or inverted membrane vesicles were prepared from purified membrane isolated from an insect cell system (Sf9 cells infected with baculovirus) expressing human MRP3, whose transport activity was validated with ATP-dependent uptake of $E_2 17\beta G$ (GenoMembrane Data Sheet). To further confirm that CLP-G is an Mrp3 substrate, the membrane vesicles were used to directly evaluate ATP-dependent uptake of CLP-G into the inverted MRP3-expressed membrane vesicles in the presence of ATP versus AMP.

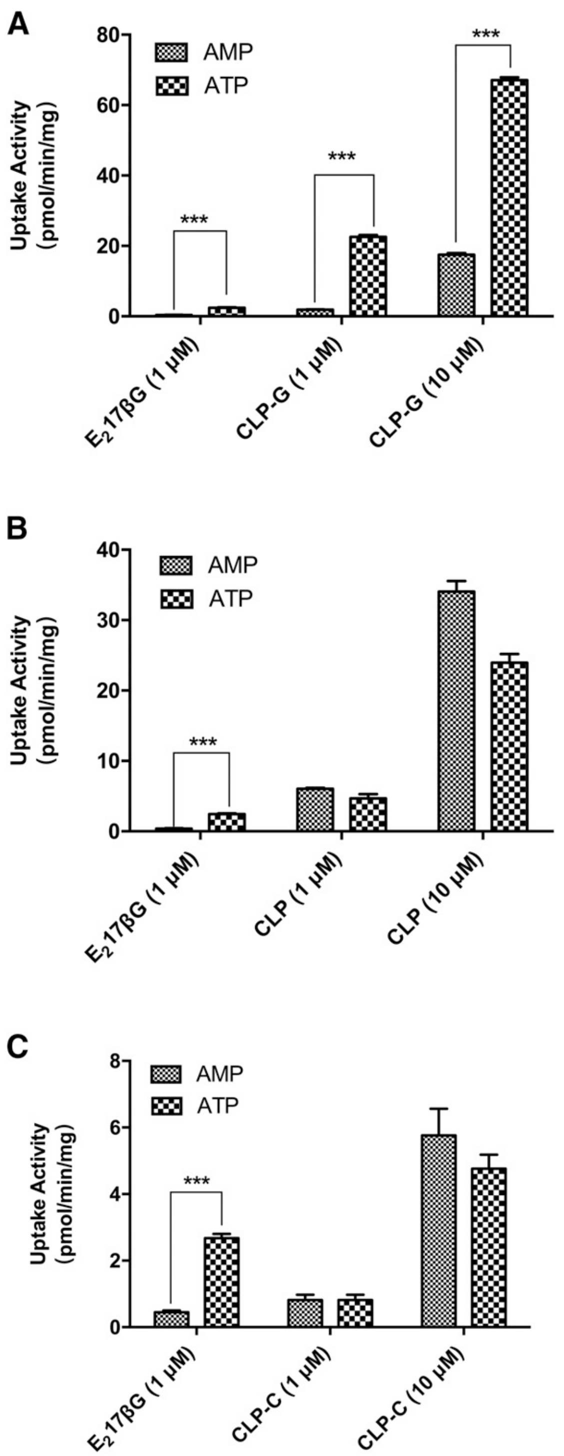


**Fig. 2.** ATP-dependent uptake of CLP-G (A), clopidogrel (B), and CLP-C (C) into inverted human recombinant MRP3 membrane vesicles. $n = 3$; $***P < 0.001$; Student's unpaired $t$ test.

As shown in Fig. 2, the uptake of $E_2 17\beta G$ was 6.5-fold higher in the presence of ATP than in the presence of AMP, indicating that the vesicular transport assay used was feasible and reliable, and that ATP-dependent uptake of $E_2 17\beta G$ into the membrane vesicles was mediated by MRP3 as described elsewhere (Shoji et al., 2004). As anticipated, at the concentrations of 1 and 10 $\mu$M, CLP-G that was taken up into the inverted MRP3 membrane vesicles was 11.7- and 3.8-fold higher in the presence of ATP than in the presence of AMP, respectively, confirming that CLP-G is indeed an Mrp3 substrate, consistent with the in vivo results.

**Clopidogrel and Its Active Metabolite Are Not an Mrp3 Substrate.** As shown in Fig. 1A, there were no significant differences in the liver-to-plasma ratios of clopidogrel or CAM between *Abcc*3 KO and WT mice, suggesting that clopidogrel and CAM are not an Mrp3 substrate in vivo. Consistent with the above findings, uptake of clopidogrel into the recombinant MRP3 inside-out membrane vesicles was not ATP dependent, as shown in Fig. 2B. These results indicated that clopidogrel and CAM are not a substrate of Mrp3.

**CLP-C Is Not an Mrp3 Substrate.** Although it is an intermediate metabolite of clopidogrel, CLP-C is glucuronidated to CLP-G principally by UGT2B7 (Ji et al., 2018). In this study, the liver-to-plasma ratio of CLP-C in *Abcc3* KO mice was approximately 37% of that in WT mice (Fig. 1A), indicating that CLP-C is not an Mrp3 substrate. Furthermore, there was no significant difference in the uptake of CLP-C into inverted human recombinant MRP3 membrane vesicles in the presence of ATP versus in the presence of AMP (Fig. 2C), suggesting a lack of MRP3-mediated, ATP-dependent uptake of CLP-C.

In summary, we reveal that CLP-G, rather than clopidogrel itself and its metabolites CLP-C and CAM, is an Mrp3 substrate. Because the glucuronidation of clopidogrel is the major elimination route from the body in humans, there are potential drug-drug interactions in patients taking clopidogrel and other substrates of MRP3 concomitantly. In addition, concurrent use of an inducer or inhibitor of MRP3 could affect the metabolism of and response to clopidogrel.

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*Participated in research design:* Xie, Ji, Tai.

*Conducted experiments:* Ji, Tai, Huang, Gu, Mi.

*Performed data analysis:* Ji, Tai, Xie.

*Wrote or contributed to the writing of the manuscript:* Xie.

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