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**Title Page:**

## Short Communication

# Mrp3 transports clopidogrel aryl glucuronide from the hepatocytes into blood

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**Running Title Page:**

1. **Running title**: Mrp3 transports clopidogrel aryl glucuronide
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   Words in the *Abstract*: 184

   Words in the *Introduction*: 500

   Words in the Results and *Discussion*: 634
4. Abbreviations used:

   Abcc3, ATP-binding cassette, subfamily C, member 3; CAM, clopidogrel active metabolite; CAMD, clopidogrel active metabolite derivative; CES, carboxylesterase; CLP, clopidogrel; CLP-C, clopidogrel carboxylate; CLP-G, clopidogrel aryl glucuronide; $E_2$17βG, estradiol-17-β-d-glucuronide; IS, internal standard; KO, knock-out; MPB, 2-bromo-3'-methoxyacetophenone; MRP, multidrug resistance-associated protein; WT, wild-type.

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## ABSTRACT

Clopidogrel aryl glucuronide (CLP-G) is a major phase II metabolite of clopidogrel generated in the liver for further excretion into urine; however, it is unknown about how CLP-G transports from hepatocytes into blood. Because MRP3 is predominantly expressed in the sinusoidal side of hepatocytes, and preferentially transports glucuronide conjugates of drug metabolites from hepatocytes into bloodstream, it was hypothesized that MRP3 could be such an efflux transporter for CLP-G. In this study, the liver-to-plasma ratios of clopidogrel and its metabolites (including CLP-G) were compared between *Abcc3* knock-out (KO) and wild-type (WT) mice, and ATP-dependent uptake of clopidogrel and CLP-G as well as estradiol-17-*β*-d-glucuronide into human recombinant MRP3 inside-out membrane vesicles was evaluated in the presence or absence of ATP. 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 μM 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. It is concluded that Mrp3 transports CLP-G from the hepatocytes into blood in an ATP-dependent manner.

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## 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 the clinical settings as an antiplatelet drug (Xie et al., 2011;Saeed et al., 2017), clopidogrel now still gains attention worldwide. Previous studies have demonstrated that clopidogrel undergoes extensive metabolism in the liver (Kazui et al., 2010;Savu et al., 2016;Tai et al., 2016;Xie et al., 2011;Zhu et al., 2013). In human body, ~ 85% of ingested clopidogrel is rapidly hydrolyzed to an immediate metabolite – clopidogrel carboxylate (CLP-C, an inactive carboxylic acid form) – by hepatic carboxylesterase 1 (CES1) (Zhu et al., 2013), whereas the remaining 15% is metabolized to clopidogrel active metabolite (CAM) by multiple cytochrome P450-mediated, two-step oxidative pathways in the liver (Kazui et al., 2010;Xie et al., 2011;2017;Savi et al., 1992;Savi et al., 2000). Furthermore, the formation of clopidogrel aryl 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 mean maximum plasma concentration of CLP-G exceeding 1000 times that of clopidogrel in patients taking this drug. If the CES1-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 enhanced antiplatelet effect as a result (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*) 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 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 the above finding, we observed that the *Abcc3* knock-out (KO) mice exhibit 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 (Zelcer et al., 2006;Kitamura et al., 2008;Kool et al., 1999;Scheffer et al., 2002) and that MRP3 preferentially transports glucuronide conjugates of drug metabolites from the hepatocytes into bloodstream (Zelcer et al., 2005;2006; Manautou et al., 2005;Smith and Dalvie, 2012), it was hypothesized that CLP-G could be transported from hepatocytes to blood via MRP3. To test the above 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 inverted membrane vesicular transport assay to directly evaluate 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.

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## 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, China. All animals were housed in an air-conditioned room with a 12-h light/ dark cycle and had free access to food and water, but were fasted for 12 h prior to studies. All the studies were approved by the Experimental Animal Welfare and Ethics Committee, Nanjing Medical University, and conducted in compliance with the Guidelines for Animal Experimentation, Nanjing Medical University, China.

**Chemicals and Reagents.** Clopidogrel (CLP) bisulfate, piroxicam (internal standard, or IS), 2-bromo-3'-methoxyacetophenone (MPB) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 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 Inc. (Toronto, Ontario, Canada). Clopidogrel carboxylic acid or carboxylate (CLP-C) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Recombinant human MRP3 inside-out membrane vesicles GM0021 (GenoMembrane, Inc., Yokohama, Kanagawa, Japan), adenosine monophosphate (AMP), adenosine triphosphate (ATP), and estradiol-17-$\beta$-d-glucuronide ($E_2$17βG) were purchased from Solvo Biotechnology (Szeged, Hungary). HPLC-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, USA).

**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 μL each) were withdrawn from the orbital venous plexus into heparinized polythene tubes pretreated with 2 μL of 500 mM MPB in acetonitrile at 10 min 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 4,000 r.p.m. for 10 min and kept frozen at − 80°C until analysis. Immediately after collecting blood samples, liver specimens were collected by sacrificing mice. Liver tissue was weighed and homogenized (20%, w/v) in normal saline solution containing 50 μL of 500 mM MPB in acetonitrile. Each sample was separated by centrifugation at 4000 r.p.m. for 10 min and kept frozen at − 80°C until analysis.

Frozen samples were thawed on ice before homogenization by vortex-mixing. Aliquots (10 μL) of plasma or liver tissue homogenates, spiked with 10 μL of piroxicam (IS) working solution (250 ng/mL), were vortex-mixed for 30 s. Protein precipitation was then performed by adding 300 μL of ice-cold acetonitrile by vortex-mixing for 3 min. Samples were centrifuged at 14,800 r.p.m. for 20 min before the supernatant was transferred to a glass vial. Aliquots of 5 μL were injected into the 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$17βG, clopidogrel, CLP-C, and CLP-G in the membrane vesicles.** As a well-characterized substrate for MRP3, $E_2$17βG was used as a positive control. ATP-dependent uptake of $E_2$17βG 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$17βG (1 μM), CLP (1 and

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10 μM each), CLP-C or CLP-G (1 and 10 μM each), respectively. Plates were pre-incubated for 5 min at 37ºC. The reactions were started by the addition of 25 μl assay buffer (Solvo kit) with ATP or AMP, allowed to proceed for 5 min at 37ºC, and terminated with 200 μl of ice-cold "washing mix" (Solvo kit). The solution was transferred to a glass fiber (Type B) filter plate (Millipore, Billerica, MA, USA) and washed 5 times with "washing mix" using a Millipore Multiscreen$^{TM}$ 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 concentrations of $E_2$17βG, CLP, CLP-C, or CLP-G in each of wells were 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$17βG, 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 ≥ 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 ± standard deviation (SD) of multiple experiments.

**Statistical analysis.** All data are expressed as mean ± SD. Student's 2-tailed, unpaired *t*-test was used for group comparisons of a single variable. A $P < 0.05$ was considered statistically significant.

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## 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 and therefore, 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 ratio of CLP-G to CLP-C was 1.8-fold higher in the liver of KO mice than in that of 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.

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$17βG (GenoMembrane Data Sheet). To further confirm that CLP-G is an Mrp3 substrate, the above 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. As shown in Fig. 2, the uptake of $E_2$17βG 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$17βG into

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the membrane vesicles was mediated by MRP3 as described elsewhere (Shoji et al., 2004). As anticipated, at the concentrations of 1 and 10 μM, 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 above *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., unpublished data). 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 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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**Disclosure of Conflict of Interests**

The authors declare no conflicts of interest.

**Authorship Contributions**

*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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**Footnote:**

1. Unnumbered footnote:

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2. The first two authors contributed equally to this work.

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**Figure legends:**

**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 vs. WT mice. n = 8; *** $P < 0.001$; Student's unpaired *t* test.

**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.

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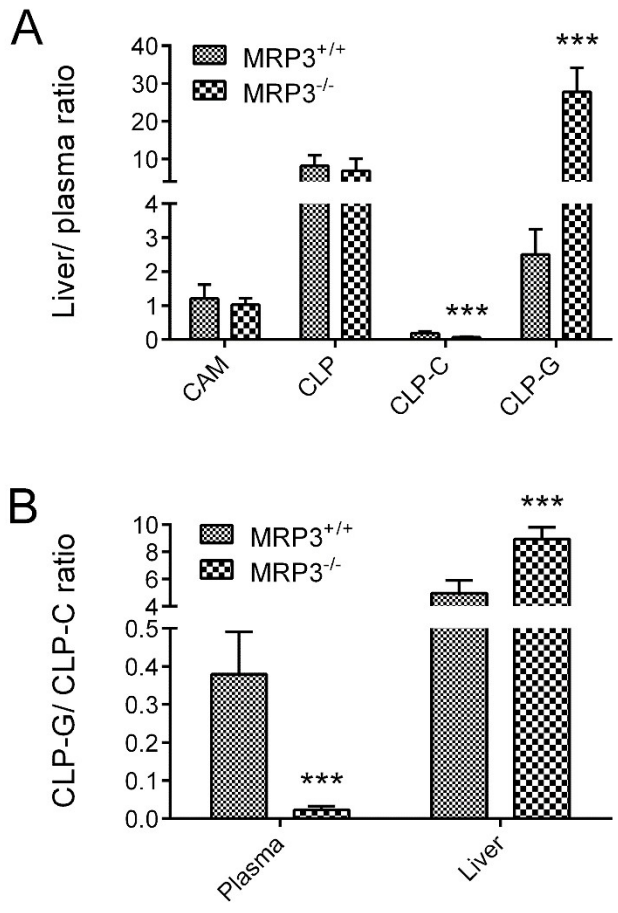


Fig. 1

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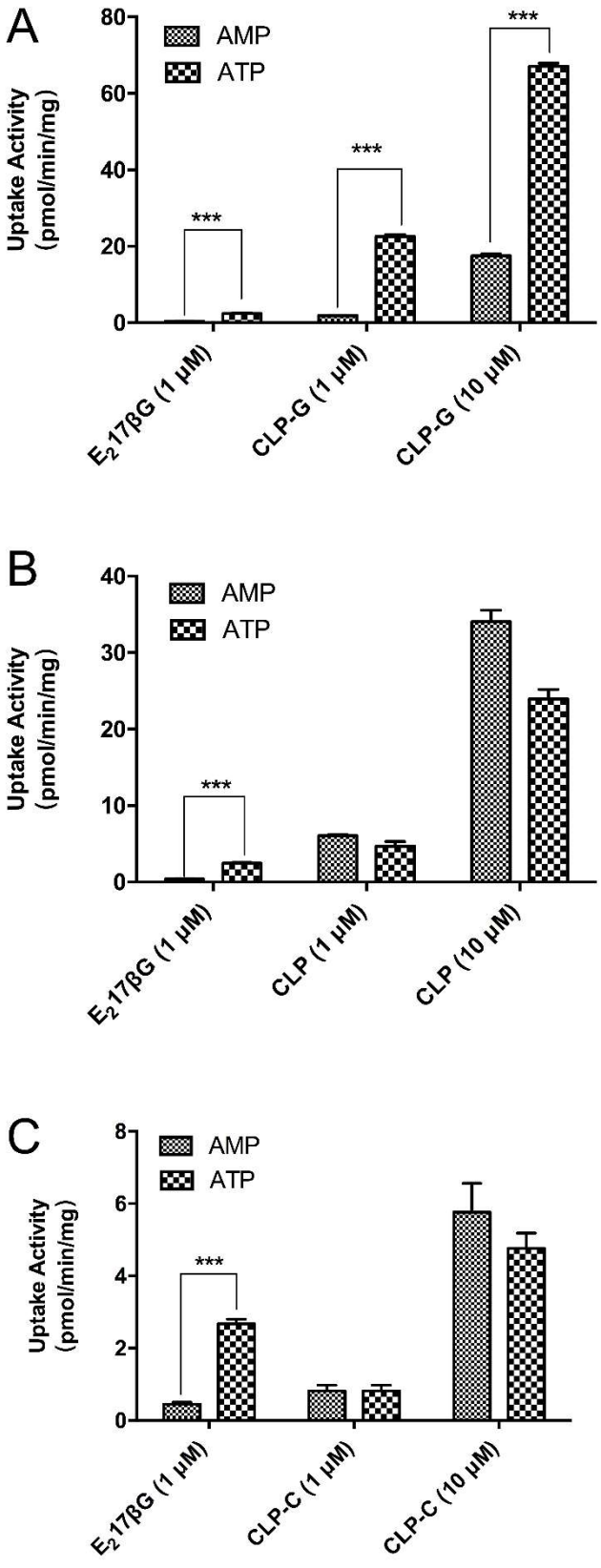


Fig. 2