

**Title Page:**

**Short Communication**

**Mrp3 transports clopidogrel aryl glucuronide from the  
hepatocytes into blood**

JIN-ZI JI, TING TAI, BEI-BEI HUANG, TONG-TONG GU, QIONG-YU MI, HONG-GUANG  
XIE

*General Clinical Research Center, Nanjing First Hospital, Nanjing Medical University (J.Z.J.,  
T.T., B.B.H., T.T.G., Q.Y.M., H.G.X.); Department of Clinical Pharmacy, School of Basic  
Medicine and Clinical Pharmacy, China Pharmaceutical University (H.G.X.); and Departments  
of Pharmacology and Clinical Pharmacy, Nanjing Medical University School of Pharmacy  
(H.G.X.), Nanjing, China*

## Running Title Page:

1. **Running title:** Mrp3 transports clopidogrel aryl glucuronide
2. **Corresponding author:** Dr. Hong-Guang Xie, General Clinical Research Center,  
Nanjing Fist Hospital, Nanjing Medical University, 68 Changle Road, Nanjing, Jiangsu  
210006, China. E-mail: [hongg.xie@gmail.com](mailto:hongg.xie@gmail.com). Tel: +85-25-52887034; fax: +86-25-  
52269924.
3. The number of --  
Text pages: 11  
Tables: 0  
Figures: 2  
References: 30  
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<sub>2</sub>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.

## **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- $\beta$ -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  $\mu$ 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. It is concluded 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 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.

## 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<sub>2</sub>17 $\beta$ G) 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  $\mu$ 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 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  $-80^{\circ}\text{C}$  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  $\mu$ L of 500 mM MPB in acetonitrile. Each sample was separated by centrifugation at 4000 r.p.m. for 10 min and kept frozen at  $-80^{\circ}\text{C}$  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 s. Protein precipitation was then performed by adding 300  $\mu$ L 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  $\mu$ L 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<sub>2</sub>17 $\beta$ G, clopidogrel, CLP-C, and CLP-G in the membrane vesicles.**

As a well-characterized substrate for MRP3, E<sub>2</sub>17 $\beta$ G was used as a positive control. ATP-dependent uptake of E<sub>2</sub>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<sub>2</sub>17 $\beta$ G (1  $\mu$ M), CLP (1 and

DMD # 78329

10  $\mu$ M each), CLP-C or CLP-G (1 and 10  $\mu$ M each), respectively. Plates were pre-incubated for 5 min at 37°C. The reactions were started by the addition of 25  $\mu$ l assay buffer (Solvo kit) with ATP or AMP, allowed to proceed for 5 min 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, USA) 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 concentrations of E<sub>2</sub>17 $\beta$ G, 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<sub>2</sub>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  $\pm$  standard deviation (SD) of multiple experiments.

**Statistical analysis.** All data are expressed as mean  $\pm$  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.



## 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 *Abcc3* 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<sub>2</sub>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<sub>2</sub>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<sub>2</sub>17βG 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 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 *Abcc3* 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.

## Acknowledgments

We thank Prof. Piet Borst, Dr. Koen van de Wetering, Sin-Ming Sit (office manager for research), Frank Hoorn (office manager for technology transfer), and Carla Rijnders, the Netherlands Cancer Institute, Amsterdam, the Netherlands, for their generously providing *Abcc3* KO mice and expert assistance.

## 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.

## References

- Backman JT, Filppula AM, Niemi M and Neuvonen PJ (2016) Role of cytochrome P450 2C8 in drug metabolism and interactions. *Pharmacol Rev* **68**:168–241.
- Bergmann TK, Filppula AM, Launiainen T, Nielsen F, Backman J and Brosen K (2016) Neurotoxicity and low paclitaxel clearance associated with concomitant clopidogrel therapy in a 60-year-old Caucasian woman with ovarian carcinoma. *Br J Clin Pharmacol* **81**:313–315.
- Floyd JS, Kaspera R, Marcianti KD, Weiss NS, Heckbert SR, Lumley T, Wiggins KL, Tamraz B, Kwok PY, Totah RA and Psaty BM (2012) A screening study of drug-drug interactions in cerivastatin users: an adverse effect of clopidogrel. *Clin Pharmacol Ther* **91**:896–904.
- Giacomini KM, Huang SM and Tweedie DJ, on behalf of the International Transporter Consortium (2010) Membrane transporters in drug development. *Nat Rev Drug Discov* **9**:215–236.
- Hirohashi T, Suzuki H and Sugiyama Y (1999) Characterization of the transport properties of cloned rat multidrug resistance-associated protein 3 (MRP3). *J Biol Chem* **274**:15181–15185.
- Itkonen MK, Tornio A, Neuvonen M, Neuvonen PJ, Niemi M and Backman JT (2016) Clopidogrel markedly increases plasma concentrations of CYP2C8 substrate pioglitazone. *Drug Metab Dispos* **44**:1364–1371.
- Kazui M, Nishiya Y, Ishizuka T, Hagihara K, Farid NA, Okazaki O, Ikeda T and Kurihara A (2010) Identification of the human cytochrome P450 enzymes involved in the two oxidative

steps in the bioactivation of clopidogrel to its pharmacologically active metabolite. *Drug Metab Dispos* **38**:92–99.

Kim SJ, Yoshikado T, Ieiri I, Maeda K, Kimura M, Irie S, Kusuhara H and Sugiyama Y (2016) Clarification of the mechanism of clopidogrel-mediated drug-drug interaction in a clinical cassette small-dose study and its prediction based on in vitro information. *Drug Metab Dispos* **44**:1622–1632.

Kitamura Y, Hirouchi M, Kusuhara H, Schuetz JD and Sugiyama Y (2008) Increasing systemic exposure of methotrexate by active efflux mediated by multidrug resistance-associated protein 3 (Mrp3/Abcc3). *J Pharmacol Exp Ther* **327**:465–473.

Kool M, van der Linden M, de Haas M, Scheffer GL, de Vree JM, Smith AJ, Jansen G, Peters GJ, Ponne N, Scheper RJ, Elferink RP, Baas F and Borst P (1999) MRP3, an organic anion transporter able to transport anti-cancer drugs. *Proc Natl Acad Sci USA* **96**:6914–6919.

Luchessi AD, Silbiger VN, Cerda A, Hirata RD, Carracedo A, Brion M, Iniguez A, Bravo M, Bastos G, Sousa AG and Hirata MH (2012) Increased clopidogrel response is associated with ABCC3 expression: a pilot study. *Clin Chim Acta* **413**:417–421.

Manautou JE, de Waart DR, Kunne C, Zelcer N, Goedken M, Borst P and Elferink RO (2005) Altered disposition of acetaminophen in mice with a disruption of the Mrp3 gene. *Hepatology* **42**:1091–1098.

Patel A, Vidula M, Kishore SP, Vedanthan R and Huffman MD (2015) Building the case for clopidogrel as a World Health Organization essential medicine. *Circ Cardiovasc Qual Outcomes* **8**:447–451.

Saeed A, Shahzad D, Faisal M, Larik FA, El-Seed HR, Channar PA (2017) Developments in the synthesis of the antiplatelet and antithrombotic drug (S)-clopidogrel. *Chirality* **29**:684–707.

Savi P, Herbert JM, Pflieger AM, Dol F, Delebassee D, Combalbert J, Defreyn G and Maffrand JP (1992) Importance of hepatic metabolism in the antiaggregating activity of the thienopyridine clopidogrel. *Biochem Pharmacol* **44**:527–532.

Savi P, Pereillo JM, Uzabiaga MF, Combalbert J, Picard C, Maffrand JP, Pascal M and Herbert JM (2000) Identification and biological activity of the active metabolite of clopidogrel. *Thromb Haemost* **84**:891–896.

Savu SN, Silvestro L, Surmeian M, Remis L, Rasit Y, Savu SR and Mircioiu C (2016) Evaluation of clopidogrel conjugation metabolism: PK studies in man and mice of clopidogrel acyl glucuronide. *Drug Metab Dispos* **44**:1490–1497.

Scheffer GL, Kool M, de Haas M, de Vree JM, Pijnenborg AC, Bosman DK, Elferink RP, van der Valk P, Borst P and Scheper RJ (2002) Tissue distribution and induction of human multidrug resistant protein 3. *Lab Invest* **82**:193–201.

Shoji T, Suzuki H, Kusuhara H, Watanabe Y, Sakamoto S and Sugiyama Y (2004) ATP-dependent transport of organic anions into isolated basolateral membrane vesicles from rat intestine. *Am J Physiol Gastrointest Liver Physiol* **287**:G749–G756.

Silvestro L, Gheorghe M, Iordachescu A, Ciuca V, Tudoroni A, Rizea SS and Tarcomnicu I (2011) Development and validation of an HPLC-MS/MS method to quantify clopidogrel acyl glucuronide, clopidogrel acid metabolite, and clopidogrel in plasma samples avoiding analyte back-conversion. *Anal Bioanal Chem* **401**:1023–1034.

Smith DA and Dalvie D (2012) Why do metabolites circulate? *Xenobiotica* **42**:107–126.

Tai T, Mi QY, Ji JZ, Yin Q, Pan YQ, Zhang MR, Huang BB and Xie HG (2016) Enhanced platelet response to clopidogrel in Abcc3-deficient mice due to its increased bioactivation. *J Cardiovasc Pharmacol* **68**:433–440.

Taubert D, Kastrati A, Harlfinger S, Gorchakova O, Lazar A, von Beckerath N, Schomig A and Schomig E (2004) Pharmacokinetics of clopidogrel after administration of a high loading dose. *Thromb Haemost* **92**:311–316

Tornio A, Filppula AM, Kailari O, Neuvonen M, Nyronen TH, Tapaninen T, Neuvonen PJ, Niemi M and Backman JT (2014) Glucuronidation converts clopidogrel to a strong time-dependent inhibitor of CYP2C8: a phase II metabolite as a perpetrator of drug-drug interactions. *Clin Pharmacol Ther* **96**:498–507.

Xie HG, Zou JJ, Hu ZY, Zhang JJ, Ye F and Chen SL (2011) Individual variability in the disposition of and response to clopidogrel: Pharmacogenomics and beyond. *Pharmacol Ther* **129**:267–289.

Xie HG, Jia YM, Tai T and Ji JZ (2017) Overcoming clopidogrel resistance: Three promising novel antiplatelet drugs developed in China. *J Cardiovasc Pharmacol* **70**:in press.

Yin Q, Tai T, Ji JZ, Mi QY, Zhang MR, Huang WJ, Cao CC and Xie HG (2016) Interleukin-10 does not modulate clopidogrel platelet response in mice. *J Thromb Haemost* **14**:596–605.

Zamek-Gliszczynski MJ, Nezasa K, Tian X, Bridges AS, Lee K, Belinsky MG, Kruh GD and Brouwer KL (2006) Evaluation of the role of multidrug resistance-associated protein (Mrp) 3

and Mrp4 in hepatic basolateral excretion of sulfate and glucuronide metabolites of acetaminophen, 4-methylumbelliferone, and harmol in Abcc3<sup>-/-</sup> and Abcc4<sup>-/-</sup> Mice. *J Pharmacol Exp Ther* **319**:1485–1491.

Zelcer N, van de Wetering K, de Waart R, Scheffer GL, Marschall HU, Wielinga PR, Kuil A, Kunne C, Smith A, van der Valk M, Wijnholds J, Elferink RO and Borst P (2006) Mice lacking Mrp3 (Abcc3) have normal bile salt transport, but altered hepatic transport of endogenous glucuronides. *J Hepatol* **44**:768–775.

Zelcer N, van de Wetering K, Hillebrand M, Sarton E, Kuil A, Wielinga PR, Tephly T, Dahan A, Beijnen JH and Borst P (2005) Mice lacking multidrug resistance protein 3 show altered morphine pharmacokinetics and morphine-6-glucuronide antinociception. *Proc Natl Acad Sci USA* **102**:7274–7279.

Zhu HJ, Wang X, Gawronski BE, Brinda BJ, Angiolillo DJ and Markowitz JS (2013) Carboxylesterase 1 as a determinant of clopidogrel metabolism and activation. *J Pharmacol Exp Ther* **344**:665–672.



**Footnote:**

1. Unnumbered footnote:

This work was supported in part by the National Natural Science Foundation of China [Grant 81473286], the Ministry of Human Resource and Social Security of China [Grant 2012-258], the Department of Science and Technology of the Province of Jiangsu [Grant BL2013001], and Nanjing First Hospital [Grant 31010300010339], China (all to Dr. Xie). In addition, Dr. Ji is a recipient of the Technology Development Training Program funded by Nanjing Medical University [Grant 2015NJMUZD044], and Dr. Xie is a recipient of the Distinguished Medical Experts of the Province of Jiangsu, China.

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

**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.

DMD # 78329

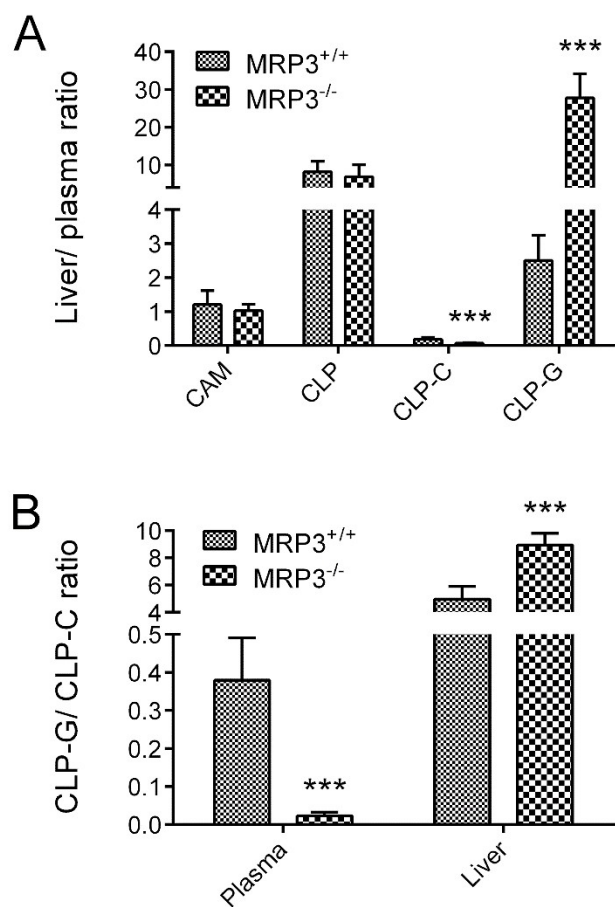


Fig. 1

DMD # 78329

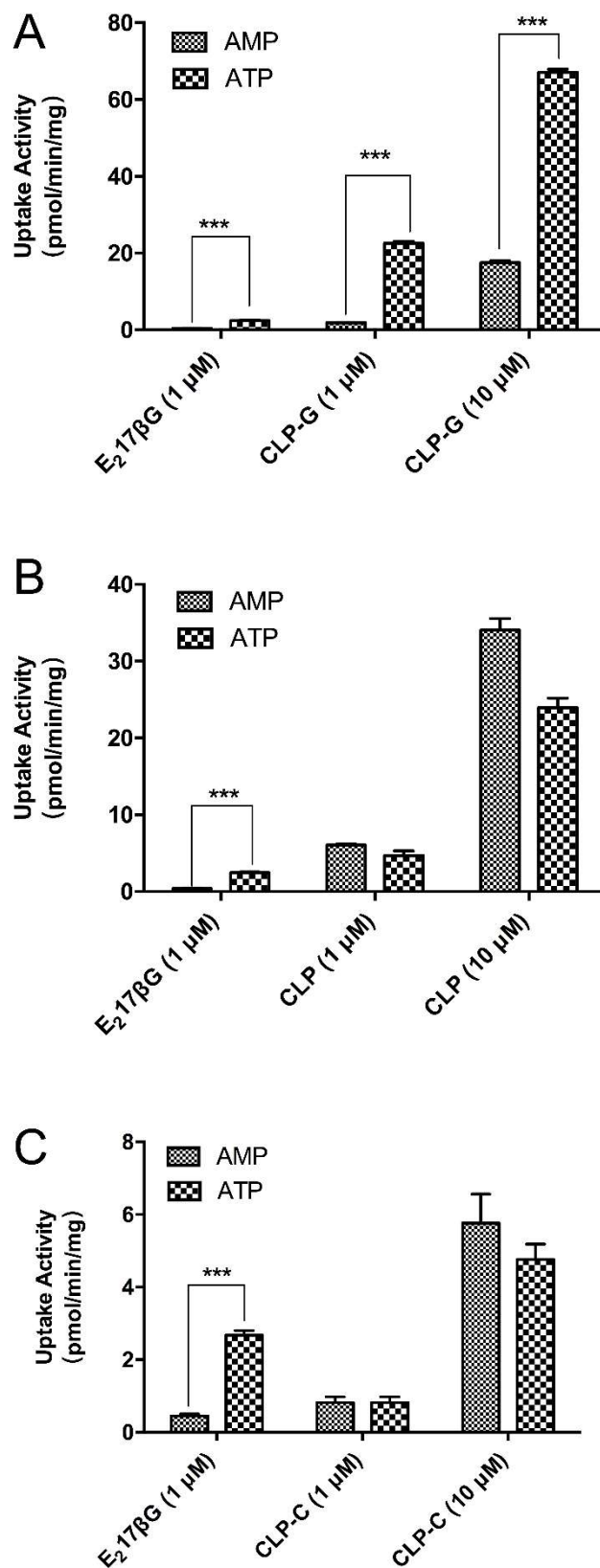


Fig. 2