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Yazun Bashir Jarrar , Dong Hyun Kim , Su-Jun Lee ,  
Jae-Gook Shin

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## **Inhibition of 20-hydroxyeicosatetraenoic acid (20-HETE) glucuronidation by non-steroidal anti-inflammatory drugs in human liver microsomes and recombinant UDP-glucuronosyltransferase enzymes**

Yazun Bashir Jarrar<sup>a,b</sup>, Dong Hyun Kim<sup>a</sup>, Su-Jun Lee<sup>a,\*</sup>, Jae-Gook Shin,<sup>a,c,\*</sup>

<sup>a</sup>Department of Pharmacology and Pharmacogenomics Research Center, Inje University College of Medicine, Inje University; <sup>b</sup>Department of Pharmacy, College of Pharmacy, Alzaytoonah University of Jordan, Amman, Jordan; <sup>c</sup>Department of Clinical Pharmacology, Inje University College of Medicine, Inje University, Busan, South Korea

### **Highlights**

- Glucuronidation of 20-HETE can be inhibited by NSAIDs
- 20-HETE glucuronidations by UGT2B7 and UGT1A9 recombinant enzymes were strongly inhibited by NSAIDs.
- Inhibition of 20-HETE glucuronidation by diclofenac was further enhanced by the genetic variant UGT2B7\*2.

**\*Correspondng authors:** Su-Jun Lee and Jae-Gook Shin

Department of Pharmacology and Pharmacogenomics Research Center, Inje University  
College of Medicine, Inje University, Busan, South Korea

E-mail address: 2sujun@inje.ac.kr (S.J.Lee), phshinj@gmail.com (J.G.Shin)

Tel: +82 51 890 8665 Fax: +82 51 895 6555

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### **Abstract**

20-hydroxyeicosatetraenoic acid (20-HETE) is an arachidonic acid metabolite which is known to increase platelet aggregation and cardiovascular risk. In this study, nine non-

steroidal anti-inflammatory drugs (NSAIDs) selected by chemical structures were screened to determine their effects on the glucuronidation of 20-HETE using human liver microsomes (HLMs). Then, the combined effects of the selected NSAID and genetic polymorphisms in UDP-glucuronosyltransferase (UGT) were investigated. Among the tested NSAIDs, diclofenac was the strongest inhibitor of 20-HETE glucuronidation with an  $IC_{50}$  value of 3.5  $\mu$ M. Celecoxib, naproxen, mefenamic acid, ibuprofen, and indomethacin showed modest inhibition with  $IC_{50}$  values of 77, 91, 190, 208, and 220  $\mu$ M, respectively, while acetylsalicylic acid, rofecoxib, and meloxicam did not inhibit 20-HETE glucuronidation. Glucuronidation of 20-HETE by UGT2B7 and UGT1A9 recombinant enzymes was significantly inhibited by indomethacin, mefenamic acid, diclofenac, ibuprofen, naproxen, and celecoxib ( $P < 0.001$ ). In addition, diclofenac exhibited a competitive inhibition mechanism with the  $K_m$  value of 20-HETE glucuronidation increasing from 23.5  $\mu$ M to 62  $\mu$ M in the presence of 3.5  $\mu$ M diclofenac. Diclofenac further decreased 20-HETE glucuronidation in HLMs carrying *UGT2B7\*2* alleles compared with the wild-type HLMs. The results from this study would be useful in understanding the alteration of 20-HETE levels in relation to NSAID and UGT genetic polymorphisms.

**Keywords:** 20-HETE; UDP-glucuronosyltransferases; Genetic polymorphisms; 20-HETE glucuronidation; Diclofenac; NSAID

## 1. Introduction

20-hydroxyeicosatetraenoic acid (20-HETE) is an arachidonic acid metabolite produced by the cytochrome P450 pathway [1]. 20-HETE increases platelet aggregation and blood pressure, decreases bleeding time, and induces diuretic effects in the kidney

[2, 3]. Higher plasma and urine levels of 20-HETE have been reported to be associated with hypertension in humans [4-6]. In addition, cardiac 20-HETE levels were increased in myocardial hypertrophy and in cardiovascular toxicity induced by doxorubicin and isoproterenol [7, 8]. Furthermore, mice plasma 20-HETE levels were elevated up to 120-fold with chronic rofecoxib treatment, which correlated with increased platelet aggregation and decreased bleeding time [2]. 20-HETE is mainly excreted through the urine after glucuronidation by UDP-glucuronosyltransferases (UGTs) [9]. Recently, we identified the UGT isoforms involved in 20-HETE glucuronidation and found UGT1A9 and UGT2B7 had a high activity for metabolizing 20-HETE [10]. In addition, we showed that *UGT2B7\*2* genetic polymorphisms altered 20-HETE glucuronidation in human liver microsomes (HLMs).

Non-steroidal anti-inflammatory drugs (NSAIDs) have been reported to cause undesirable cardiovascular side effects and toxicities [11]. In many countries, diclofenac and rofecoxib consistently rank the highest in terms of cardiovascular risk [12]. Rofecoxib has been banned from the market due to its cardiovascular side effect and pharmacovigilance studies showed that diclofenac has a high cardiovascular toxicity similar to rofecoxib [13]. Studies showed that both diclofenac and rofecoxib increased 20-HETE plasma levels [2, 14]. 20-HETE-glucuronide levels were decreased in urine samples of patients after indomethacin treatment [15]. However, how NSAIDs alter 20-HETE levels remains unclear. NSAIDs are excreted in the urine in the glucuronidated form [16, 17]. Furthermore, NSAIDs have been shown to inhibit multiple UGT isoforms, including UGT2B7 and UGT1A9 [18, 19]. Information regarding the effects of NSAIDs on the inhibition of 20-HETE glucuronidation is lacking. Therefore, in this study, we investigated

whether commonly used NSAIDs inhibit 20-HETE glucuronidation, and presented the potency of inhibition as quantitative values using pooled HLMs and recombinant UGT enzymes.

## 2. Materials and Methods

### 2.1. Chemicals and reagents

Alamethicin and uridine diphosphoglucuronic acid (UDPGA) were obtained from Sigma Aldrich (St. Louis, MO, USA). 20-HETE was obtained from Cayman Chemical (Ann Arbor, MI, USA). The internal standard, estrone-glucuronide, was purchased from Toronto Research Chemicals (Toronto, Canada). The NSAIDs, diclofenac, ibuprofen, mefenamic acid, acetylsalicylic acid, naproxen, indomethacin, meloxicam, celecoxib, and rofecoxib were purchased from Sigma Aldrich (St. Louis, MO, USA). All other chemicals and organic solvents were of the highest grade available from commercial sources.

### 2.2. Human liver microsomes

Pooled HLM was obtained from BD Gentest (H161; BD Gentest, Woburn, MA, USA) to test the inhibition activity of NSAIDs against 20-HETE glucuronidation. Further information about the commercial pooled HLM is available on the website of the company (<https://www.bdbiosciences.com/en-eu>). In order to test the inter-individual variation in 20-HETE glucuronidation in relation to diclofenac inhibition and *UGT2B7\*2* genotype, HLMs derived from 44 Korean donors ( $48.7 \pm 10.7$  years old; mean  $\pm$  SD, 23 females and 21 males) were obtained from the Biobank at Inje Pharmacogenomics Research Center (Inje University College of Medicine, Busan, Korea) [10]. The research

protocol for the use of HLMs was approved by the institutional review board of Busan Paik Hospital (Busan, Korea) [20].

### 2.3. *In vitro* glucuronidation

*In vitro* 20-HETE glucuronidation was performed as described previously [10]. Briefly, 15  $\mu\text{g}$  of HLMs was reconstituted with a reaction mixture containing 1–500  $\mu\text{M}$  20-HETE and 1.5 mg/mL alamethicin in 0.5 M Tris-HCl buffer (pH 7.4). The reaction was initiated by the addition of 5 mM UDPGA and incubation at 37°C for 30 min. Then, the reaction was terminated by the addition of 100  $\mu\text{L}$  of acetonitrile containing 1.2  $\mu\text{M}$  estrone-glucuronide as the internal standard. The samples were kept at –20°C until analysis. For quantitative analysis of 20-HETE-glucuronide and the internal standard, an API 3000 liquid chromatography/tandem mass spectrometry (LC-MS/MS) system (Applied Biosystems, Foster City, CA, USA) coupled with a 1100 series high-performance liquid chromatography system (Agilent Technologies, Santa Clara, CA, USA) was used as described previously [10]. The  $K_m$  and  $V_{max}$  values of 20-HETE glucuronidation in pooled HLMs were estimated using Sigma plot 12.1 software (Systat Software, Inc., San Jose, CA, USA).

### 2.4. *Inhibition* assays

To determine the effects of NSAIDs on 20-HETE glucuronidation, seven different concentrations from each NSAID ranging from 0.1 to 1000  $\mu\text{M}$  were incubated with 15  $\mu\text{g}$  of pooled HLMs, before addition of 10  $\mu\text{M}$  20-HETE, under similar incubation conditions as described above. Nine different NSAIDs were used: diclofenac, ibuprofen, mefenamic acid, acetylsalicylic acid, naproxen, indomethacin, meloxicam, celecoxib,

and rofecoxib, representing different chemical structural groups and cyclooxygenase selectivity as shown in Table 1. The general UGT inhibitor, probenecid, was used as a positive control of glucuronidation inhibition [21, 22]. The  $IC_{50}$  value was calculated using Sigma plot software. To determine the inhibition effects of NSAIDs on 20-HETE glucuronidation by UGT1A9 and UGT2B7 isozymes, diclofenac, ibuprofen, mefenamic acid, naproxen, indomethacin, and celecoxib were incubated with 15  $\mu$ M recombinant UGT1A9 and UGT2B7 enzymes under similar *in vitro* 20-HETE glucuronidation conditions described above. The NSAID concentrations were  $IC_{50}$  values estimated from the NSAID inhibition values of 20-HETE glucuronidation in pooled HLMs (Table 1). The mechanism of diclofenac inhibition of 20-HETE glucuronidation was identified by incubating 3.5  $\mu$ M diclofenac with pooled HLMs and with various 20-HETE concentrations (1–500  $\mu$ M) under the same reaction conditions described above. The levels of 20-HETE-glucuronide were compared with pooled HLM samples not incubated with diclofenac. All values represent the means  $\pm$  standard deviation (SD) of triplicate reactions.

### 2.5. *UGT2B7\*2* genotype

Because we previously showed that a non-synonymous *UGT2B7\*2* 802C>T genetic variant affected *in vitro* 20-HETE glucuronidation [10], the effect of *UGT2B7\*2* on the inhibition of 20-HETE glucuronidation by diclofenac was investigated. In this study, genotyping of *UGT2B7\*2* in 24 HLMs was performed using a previously described DNA sequencing method [10].

## 2.6. Statistical Analyses

The results of the inhibitory effects of NSAIDs on 20-HETE glucuronidation rate were analyzed using two-tailed Student's *t*-test. All statistical analyses were performed using the SAS program (version 9.1.3; SAS Institute, Cary, NC, USA). Statistically significant changes compared with the control groups were indicated as \**P* < 0.05, \*\**P* < 0.01, and \*\*\**P* < 0.001.

## 3. Results

The *in vitro* metabolism of 20-HETE by a set of pooled HLMs produced 20-HETE glucuronidation resulting in  $K_m$  and  $V_{max}$  values of 23.5  $\mu\text{M}$  and 2  $\mu\text{M}/\text{min}/\text{mg}$ , respectively (Figure 1). Then, 10  $\mu\text{M}$  of 20-HETE (about half the determined  $K_m$  value, 23.5  $\mu\text{M}$ ) was used to investigate the NSAID inhibition on 20-HETE glucuronidation. Diclofenac was the strongest inhibitor of 20-HETE glucuronidation with an  $\text{IC}_{50}$  value of 3.5  $\mu\text{M}$  (Figure 2). Celecoxib, naproxen, mefenamic acid, ibuprofen, and indomethacin showed modest inhibition with  $\text{IC}_{50}$  values of 77, 91, 190, 208, and 220  $\mu\text{M}$ , respectively, while acetylsalicylic acid, rofecoxib, and meloxicam did not inhibit 20-HETE glucuronidation. The positive control inhibitor, probenecid, exhibited an  $\text{IC}_{50}$  of 222  $\mu\text{M}$ . All  $\text{IC}_{50}$  values for the tested NSAIDs against 20-HETE glucuronidation are presented in Table 1. As shown in Figure 3, diclofenac, celecoxib, naproxen, mefenamic acid, ibuprofen, and indomethacin significantly inhibited 20-HETE glucuronidation by the main 20-HETE metabolizing UGT isoforms, UGT1A9 and UGT2B7 (*P* < 0.001). Next, we identified the inhibitory mechanism of 3.5  $\mu\text{M}$  diclofenac, the strongest inhibitor of

20-HETE glucuronidation. The  $K_m$  value of 20-HETE glucuronidation in pooled HLMs increased significantly ( $P < 0.05$ ) from 23.5 to 62  $\mu\text{M}$ , and in the presence of diclofenac, the  $V_{\text{max}}$  value did not change, as shown in Figure 4.

The degree of 20-HETE glucuronidation inhibition by diclofenac differed among the tested HLMs. The inhibition ranged from 40% to 75% among 10 different HLMs when using 3.5  $\mu\text{M}$  diclofenac. Because the *UGT2B7\*2* genetic variant decreased the capacity of HLMs for 20-HETE glucuronidation [10], we investigated the 20-HETE glucuronidation rate in HLMs carrying the *UGT2B7\*2* genetic variant in the presence of 3.5  $\mu\text{M}$  diclofenac (Fig. 5). The HLMs having the homozygous *UGT2B7\*2* genotype incubated with diclofenac exhibited significantly decreased 20-HETE glucuronide levels compared with the *UGT2B7* wild type incubated with diclofenac ( $P < 0.05$ ) and the *UGT2B7* wild type incubated without diclofenac ( $P < 0.01$ ).

#### 4. Discussion and conclusion

Alteration of 20-HETE metabolism can disturb the homeostasis of 20-HETE level, which may cause cardiovascular diseases and drug-induced cardiotoxicities [1]. In this study, a novel interaction mechanism between NSAID and 20-HETE *via* UGT enzymes was suggested. Inhibition of 20-HETE glucuronidation may increase free 20-HETE level which may be associated with the risk of cardiovascular disease at least in part. We used nine different NSAIDs classified based on different chemical group structures in the present study. Diclofenac, the acetic acid derivative, was the strongest inhibitor of 20-

HETE glucuronidation. The  $IC_{50}$  value of diclofenac (3.5  $\mu$ M) was 1.6% (64-fold strong inhibition) of the value of general UGT inhibitor probenecid (222  $\mu$ M). The cardiotoxicity of diclofenac has been reported [12] and it was reported that plasma 20-HETE levels were increased after diclofenac treatment in a group of volunteers [14]. The results from this study indicated that diclofenac may clinically alter 20-HETE levels by inhibiting its glucuronidation. Further clinical investigations are needed to study the effects of 20-HETE glucuronidation inhibition caused by diclofenac on plasma and urine 20-HETE levels and cardiovascular events. Other NSAIDs with clinically reported cardiovascular events, such as ibuprofen and mefenamic acid, showed moderate inhibition of 20-HETE glucuronidation with  $IC_{50}$  values ranging from 190 to 208  $\mu$ M. Inhibition of 20-HETE metabolism by these NSAIDs may play a role in their cardiovascular toxicities, thus, further clinical studies are needed. Rofecoxib was withdrawn from the market due to its cardiovascular side effect [23]. A previous study showed that chronic treatment of mice with rofecoxib increased the plasma 20-HETE levels up to 120-fold which was associated with decreased bleeding time; however, increased expression of 20-HETE-synthesizing P450 cyp4a was not observed [2]. Because rofecoxib is glucuronidated by UGT2B15, we hypothesized that rofecoxib may inhibit or affect the UGT metabolism of 20-HETE. However, inhibition of 20-HETE glucuronidation by rofecoxib was not observed, indicating that the synthesis or the metabolism of 20-HETE glucuronidation might not be affected by rofecoxib. Therefore, another mechanism may be involved in this endogenous drug interaction, such as inhibition of 20-HETE metabolism by Cox-2, which was reported to be involved in 20-HETE metabolism [24]. Cox-2 levels are low in the normal state but are induced significantly in the inflammatory state [25]. Therefore, in inflammatory situations, Cox-2 and UGT may be

involved in the 20-HETE elimination pathways, however, in the normal state, UGT may be the major 20-HETE elimination pathway. Whether rofecoxib affects the 20-HETE metabolism by Cox-2 is unclear. The UGT1A9 and UGT2B7 isoforms were identified as major metabolizers of 20-HETE [10]. Because 20-HETE is metabolized by multiple UGT isoforms, inhibition of 20-HETE glucuronidation in pooled HLMs requires inhibition of multiple UGT isoforms, including UGT1A9 and UGT2B7. In this study, both UGT1A9 and UGT2B7 were significantly inhibited by the tested NSAIDs, consistent with previous studies showing multiple UGT isoforms were inhibited by NSAIDs [18, 19]. Reportedly, NSAIDs inhibit UGTs by both competitive and non-competitive mechanisms [26, 27]. In this study, we selected the strongest 20-HETE glucuronidation inhibitor, diclofenac, and showed that the affinity, represented by the  $K_m$  value, increased from 23.5 to 67  $\mu\text{M}$  in the presence of 3.5  $\mu\text{M}$  diclofenac, while the  $V_{\text{max}}$  value did not change. Based on these results, both diclofenac and 20-HETE may compete for the same UGT enzyme binding site in the glucuronidation reaction.

The *UGT2B7\*2* genetic variant affects the metabolism of drugs and endogenous compounds including diclofenac and 20-HETE [10, 28]. Reportedly, *UGT2B7\*2* is associated with diclofenac-induced hepatotoxicity [29]. Because variation in the 20-HETE inhibition by diclofenac among the tested HLMs was observed in this study and we previously reported that *UGT2B7* influenced 20-HETE glucuronidation, we investigated the possible influence of the *UGT2B7\*2* genetic variant on 20-HETE glucuronidation in HLMs incubated with diclofenac. *UGT2B7\*2 802C>T* had an additive effect with diclofenac in decreasing 20-HETE glucuronidation. Liver tissues having *UGT2B7\*2* genotype treated with diclofenac exhibited significantly decreased 20-HETE glucuronidation compared with

the wild-type UGT2B7. Consequently, individuals carrying *UGT2B7\*2* alleles and treated with diclofenac may have further altered cardio toxic 20-HETE levels.

In conclusion, this is the first study showing that multiple NSAIDs inhibited glucuronidation of 20-HETE in HLMs and recombinant UGT enzymes. Among nine NSAIDs, diclofenac was found to exert the strongest inhibition on 20-HETE glucuronidation. Inhibition of 20-HETE glucuronidation by diclofenac was further enhanced in the HLMs carrying a *UGT2B7\*2* genotype. These findings would increase our understanding of the NSAID-related variations in 20-HETE levels with cardiovascular complications in future studies.

### **Author Statement**

Yazun Bashir Jarrar: Experimental investigation, Methodology, Software analysis, Original draft writing. Dong Hyun Kim: Chemical analysis, Methodology, Data curation. Su-Jun Lee: Conceptualization, Methodology, Supervision, Original draft preparation, reviewing and editing. Jae-Gook Shin: Supervision, Reviewing and data validation.

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## References

- [1] O.H. Elshenawy, A. Anwar-Mohamed, A.O. El-Kadi, 20-Hydroxyeicosatetraenoic acid is a potential therapeutic target in cardiovascular diseases, *Curr. Drug Metab.* 14 (2013) 706-719.
- [2] J.Y. Liu, N. Li, J. Yang, H. Qiu, D. Ai, N. Chiamvimonvat, Y. Zhu, B.D. Hammock, Metabolic profiling of murine plasma reveals an unexpected biomarker in rofecoxib-mediated cardiovascular events, *Proc. Natl. Acad. Sci. U S A.* 107 (2010) 17017-17022.
- [3] C.L. Laffer, M. Laniado-Schwartzman, M.H. Wang, A. Nasjletti, F. Eljovich, 20-HETE and furosemide-induced natriuresis in salt-sensitive essential hypertension, *Hypertension* 41 (2003) 703-708.
- [4] H. Liu, Y. Zhao, D. Nie, J. Shi, L. Fu, Y. Li, D. Yu, J. Lu, Association of a functional cytochrome P450 4F2 haplotype with urinary 20-HETE and hypertension, *J. Am. Soc. Nephrol.* 19 (2008) 714-721.
- [5] N.C. Ward, I.J. Tsai, A. Barden, F.M. van Bockxmeer, I.B. Puddey, J.M. Hodgson, K.D. Croft, A single nucleotide polymorphism in the CYP4F2 but not CYP4A11 gene is associated with increased 20-HETE excretion and blood pressure, *Hypertension* 51 (2008) 1393-1398.

- [6] K. Inoue, K. Sodhi, N. Puri, K.H. Gotlinger, J. Cao, R. Rezzani, J.R. Falck, N.G. Abraham, M. Laniado-Schwartzman, Endothelial-specific CYP4A2 overexpression leads to renal injury and hypertension via increased production of 20-HETE, *Am. J. Physiol. Renal. Physiol.* 297 (2009) F875-884.
- [7] H.N. Althurwi, O.H. Elshenawy, A.O. El-Kadi, Fenofibrate modulates cytochrome P450 and arachidonic acid metabolism in the heart and protects against isoproterenol-induced cardiac hypertrophy, *J. Cardiovasc. Pharmacol.* 63 (2014) 167-177.
- [8] B.N. Zordoky, A. Anwar-Mohamed, M.E. Aboutabl, A.O. El-Kadi, Acute doxorubicin cardiotoxicity alters cardiac cytochrome P450 expression and arachidonic acid metabolism in rats, *Toxicol. Appl. Pharmacol.* 242 (2010) 38-46.
- [9] C. Prakash, J.Y. Zhang, J.R. Falck, K. Chauhan, I.A. Blair, 20-Hydroxyeicosatetraenoic acid is excreted as a glucuronide conjugate in human urine, *Biochem. Biophys. Res. Commun.* 185 (1992) 728-733.
- [10] Y.B. Jarrar, E.Y. Cha, K.A. Seo, J.L. Ghim, H.J. Kim, D.H. Kim, S.J. Lee, J.G. Shin, Determination of major UDP-glucuronosyltransferase enzymes and their genotypes responsible for 20-HETE glucuronidation, *J. Lipid Res.* 55 (2014) 2334-2342.
- [11] P.L. Temporelli, G.B. Zito, R.F. Pedretti, F.I. Belisarii, G. Putorti, P. Faggiano, [Nonsteroid anti-inflammatory drugs (NSAID) and risk of cardiovascular events. Literature review and clinical implications], *Monaldi. Arch. Chest. Dis.* 82 (2014) 165-170.

- [12] P. McGettigan, D. Henry, Use of non-steroidal anti-inflammatory drugs that elevate cardiovascular risk: an examination of sales and essential medicines lists in low-, middle-, and high-income countries, *PLoS Med.* 10 (2013) e1001388.
- [13] E.L. Fosbol, F. Folke, S. Jacobsen, J.N. Rasmussen, R. Sorensen, T.K. Schramm, S.S. Andersen, S. Rasmussen, H.E. Poulsen, L. Kober, C. Torp-Pedersen, G.H. Gislason, Cause-specific cardiovascular risk associated with nonsteroidal antiinflammatory drugs among healthy individuals, *Circ. Cardiovasc. Qual. Outcomes* 3 (2010) 395-405.
- [14] M.J. van Erk, S. Wopereis, C. Rubingh, T. van Vliet, E. Verheij, N.H. Cnubben, T.L. Pedersen, J.W. Newman, A.K. Smilde, J. van der Greef, H.F. Hendriks, B. van Ommen, Insight in modulation of inflammation in response to diclofenac intervention: a human intervention study, *BMC Med. Genomics* 3 (2010) 5.
- [15] B. Watzer, S. Reinalter, H.W. Seyberth, H. Schweer, Determination of free and glucuronide conjugated 20-hydroxyarachidonic acid (20-HETE) in urine by gas chromatography/negative ion chemical ionization mass spectrometry, *Prostaglandins Leukot. Essent. Fatty Acids* 62 (2000) 175-181.
- [16] G.E. Kuehl, J.W. Lampe, J.D. Potter, J. Bigler, Glucuronidation of nonsteroidal anti-inflammatory drugs: identifying the enzymes responsible in human liver microsomes, *Drug Metab. Dispos.* 33 (2005) 1027-1035.
- [17] S. Kumar, K. Samuel, R. Subramanian, M.P. Braun, R.A. Stearns, S.H. Chiu, D.C. Evans, T.A. Baillie, Extrapolation of diclofenac clearance from in vitro microsomal metabolism data: role of acyl glucuronidation and sequential oxidative metabolism of the acyl glucuronide, *J. Pharmacol. Exp. Ther.* 303 (2002) 969-978.

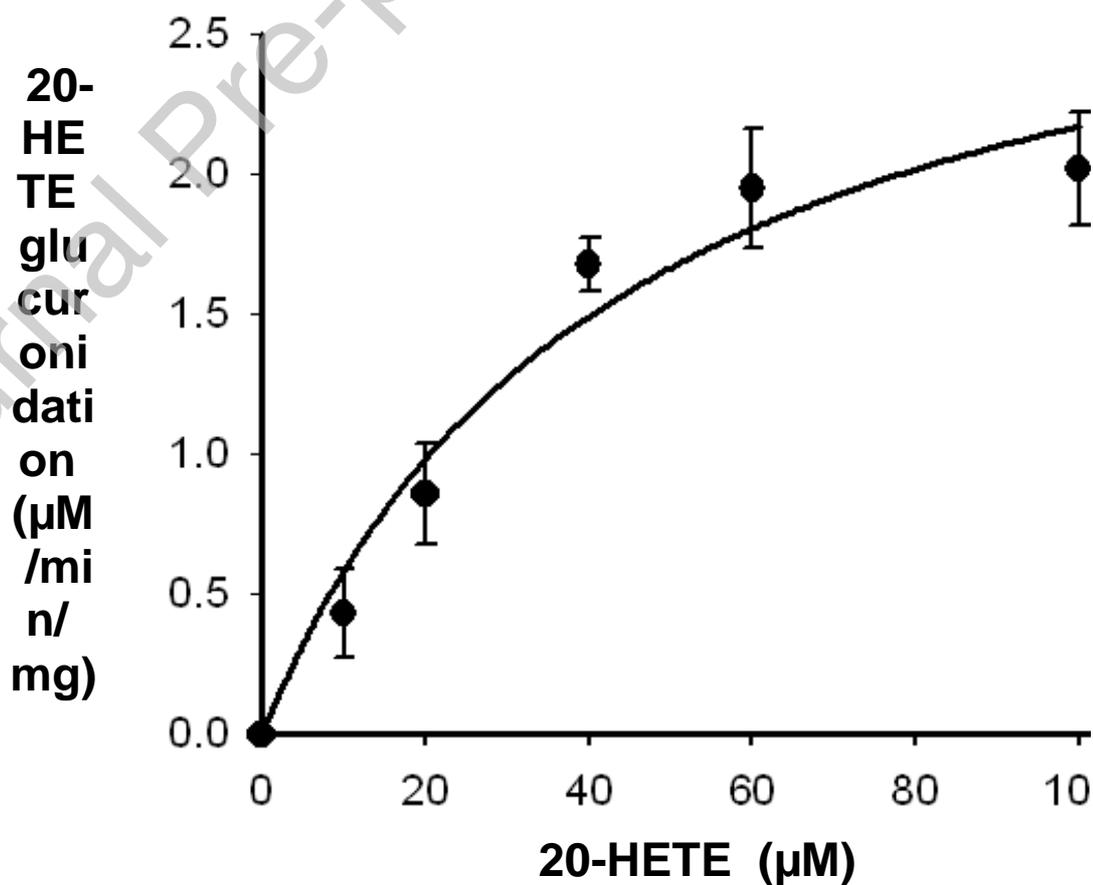
- [18] Y. Mano, T. Usui, H. Kamimura, In vitro inhibitory effects of non-steroidal anti-inflammatory drugs on 4-methylumbelliferone glucuronidation in recombinant human UDP-glucuronosyltransferase 1A9--potent inhibition by niflumic acid, *Biopharm. Drug Dispos.* 27 (2006) 1-6.
- [19] Y. Mano, T. Usui, H. Kamimura, Inhibitory potential of nonsteroidal anti-inflammatory drugs on UDP-glucuronosyltransferase 2B7 in human liver microsomes, *Eur. J. Clin. Pharmacol.* 63 (2007) 211-216.
- [20] S.J. Lee, S.S. Lee, H.J. Jung, H.S. Kim, S.J. Park, C.W. Yeo, J.G. Shin, Discovery of novel functional variants and extensive evaluation of CYP2D6 genetic polymorphisms in Koreans, *Drug Metab. Dispos.* 37 (2009) 1464-1470.
- [21] A. Mendonza, I. Hanna, D. Meyers, P. Koo, S. Neelakantham, B. Zhu, T. Majumdar, S. Rebello, G. Sunkara, J. Chen, Assessment of pharmacokinetic drug-drug interaction between pradigastat and atazanavir or probenecid, *J. Clin. Pharmacol.* 56 (2016) 355-64.
- [22] S.M. Huang, J.M. Strong, L. Zhang, K.S. Reynolds, S. Nallani, R. Temple, S. Abraham, S.A. Habet, R.K. Baweja, G.J. Burckart, S. Chung, P. Colangelo, D. Frucht, M.D. Green, P. Hepp, E. Karnaukhova, H.S. Ko, J.I. Lee, P.J. Marroum, J.M. Norden, W. Qiu, A. Rahman, S. Sobel, T. Stifano, K. Thummel, X.X. Wei, S. Yasuda, J.H. Zheng, H. Zhao, L.J. Lesko, New era in drug interaction evaluation: US Food and Drug Administration update on CYP enzymes, transporters, and the guidance process, *J. Clin. Pharmacol.* 48 (2008) 662-670.
- [23] L.G. Howes, Selective COX-2 inhibitors, NSAIDs and cardiovascular events - is celecoxib the safest choice?, *Ther. Clin. Risk Manag.* 3 (2007) 831-845.

- [24] D.H. Kim, N. Puri, K. Sodhi, J.R. Falck, N.G. Abraham, J. Shapiro, M.L. Schwartzman, Cyclooxygenase-2 dependent metabolism of 20-HETE increases adiposity and adipocyte enlargement in mesenchymal stem cell-derived adipocytes, *J. Lipid Res.* 54 (2013) 786-793.
- [25] E. Ricciotti, G.A. FitzGerald, Prostaglandins and inflammation, *Arterioscler. Thromb. Vasc. Biol.* 31 (2011) 986-1000.
- [26] J.O. Miners, K. Bowalgaha, D.J. Elliot, P. Baranczewski, K.M. Knights, Characterization of niflumic acid as a selective inhibitor of human liver microsomal UDP-glucuronosyltransferase 1A9: application to the reaction phenotyping of acetaminophen glucuronidation, *Drug Metab. Dispos.* 39 (2011) 644-652.
- [27] K.M. Knights, L.K. Winner, D.J. Elliot, K. Bowalgaha, J.O. Miners, Aldosterone glucuronidation by human liver and kidney microsomes and recombinant UDP-glucuronosyltransferases: inhibition by NSAIDs, *Br. J. Clin. Pharmacol.* 68 (2009) 402-412.
- [28] H. Wang, L. Yuan, S. Zeng, Characterizing the effect of UDP-glucuronosyltransferase (UGT) 2B7 and UGT1A9 genetic polymorphisms on enantioselective glucuronidation of flurbiprofen, *Biochem. Pharmacol.* 82 (2011) 1757-1763.
- [29] A.K. Daly, G.P. Aithal, J.B. Leathart, R.A. Swainsbury, T.S. Dang, C.P. Day, Genetic susceptibility to diclofenac-induced hepatotoxicity: contribution of UGT2B7, CYP2C8, and ABCC2 genotypes, *Gastroenterology* 132 (2007) 272-281.

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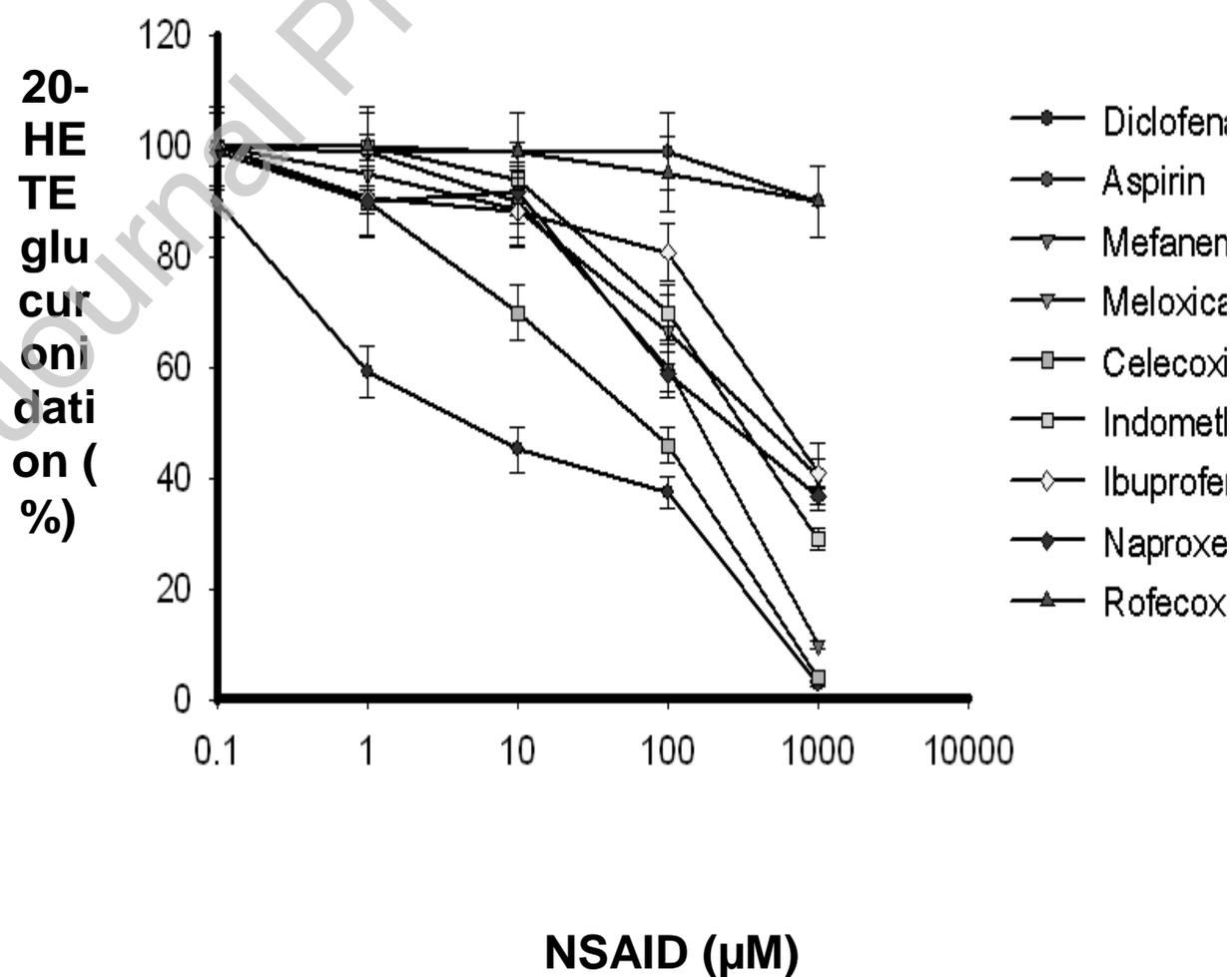
**Figure legends**

**Fig. 1.** Dose-dependent 20-HETE glucuronidation by human liver microsomes. Reaction mixture included human liver microsomes (15  $\mu\text{g}$ ), various concentrations of 20-HETE (1–100  $\mu\text{M}$ ), and 5 mM UDPGA in 0.5 M Tris-HCl buffer (pH 7.4). Other detailed experimental conditions and detection methods are described under Materials & Methods. The  $K_m$  and  $V_{\text{max}}$  values for the 20-HETE glucuronidation were 23.5  $\mu\text{M}$  and 2  $\mu\text{M}/\text{min}/\text{mg}$ , respectively.

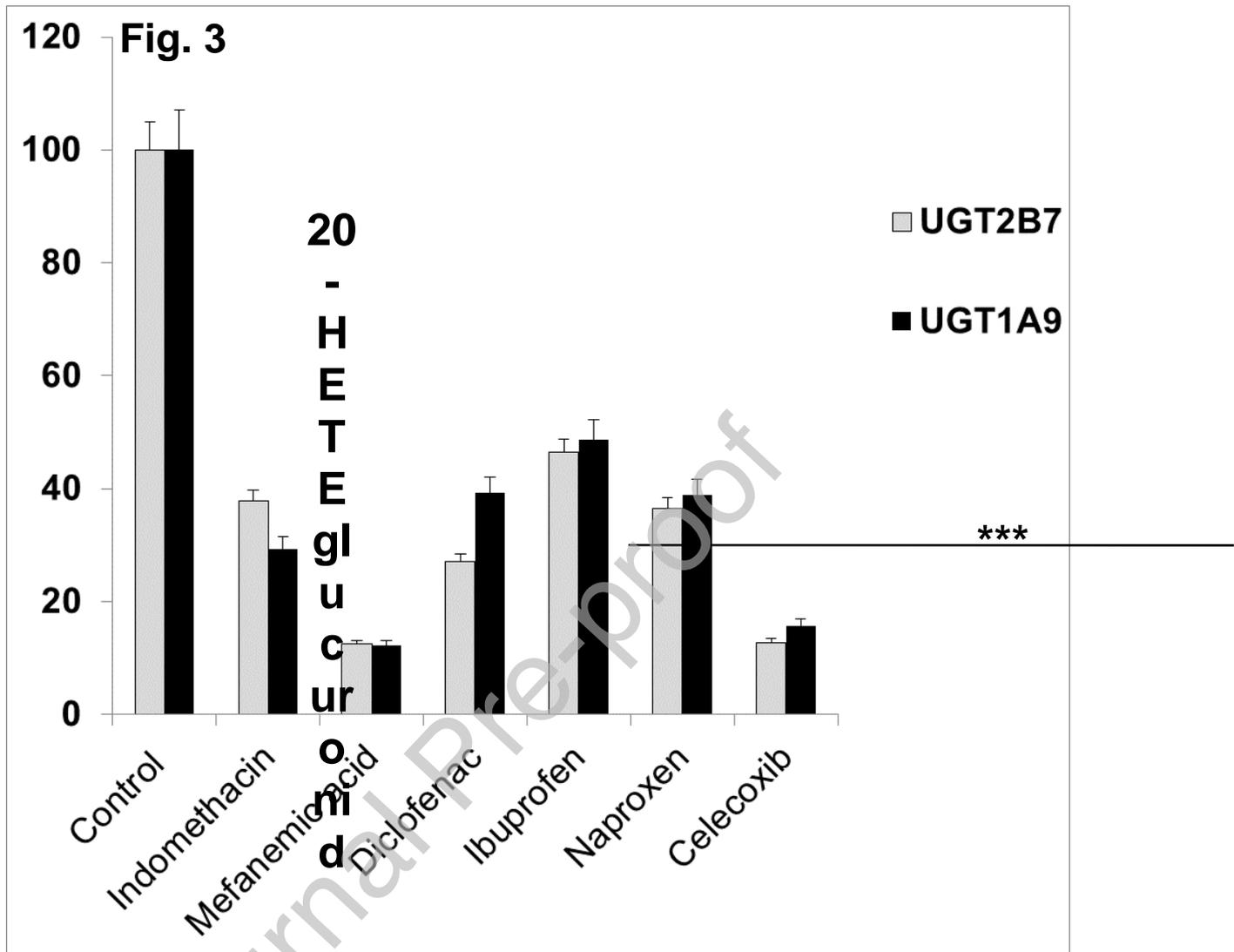
**Fig. 1**

**Fig. 2.** Inhibition of 20-HETE glucuronidation by NSAIDs. Human liver microsomes (15 ug) were reconstituted with a reaction mixture containing 10  $\mu$ M 20-HETE in 0.5 M Tris-HCl buffer (pH 7.4) at 37°C for 30 min. The concentration of the NSAIDs used ranged from 0.1 to 1000  $\mu$ M. Experimental conditions, detection and other details are described under Materials & Methods. Diclofenac was the strongest inhibitor of 20-HETE glucuronidation with an  $IC_{50}$  value of 3.5  $\mu$ M. Celecoxib, naproxen, mefenamic acid, ibuprofen, and indomethacin showed modest inhibition, while aspirin, rofecoxib, and meloxicam did not inhibit 20-HETE glucuronidation.

**Fig. 2**



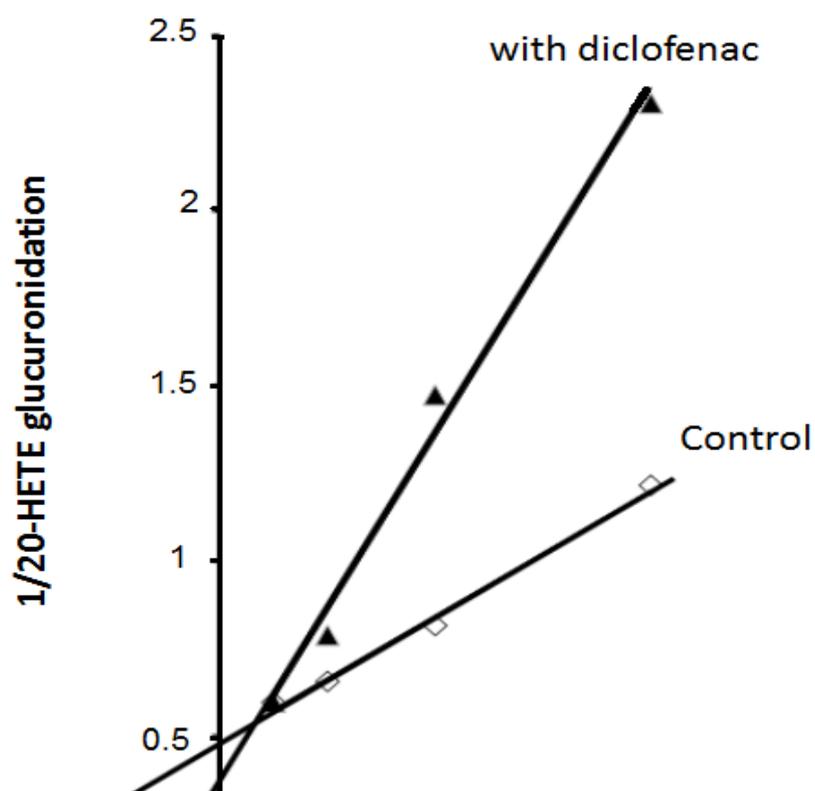
**Fig. 3.** Inhibition of 20-HETE glucuronidation by NSAIDs in recombinant UGT1A9 and UGT2B7 enzymes. The NSAIDs were incubated with 15  $\mu$ M of recombinant UGT2B7 and YGT1A9. The NSAID concentrations used were  $IC_{50}$  values estimated from the inhibition values of 20-HETE glucuronidation in human liver microsomes (Table 1). Details are described under Materials and Methods. All the tested NSAIDs inhibited UGT1A9 and UGT2B7, the major 20-HETE metabolizing UGT isoforms. Statistically significant differences compared with controls are indicated as \*\*\* $P < 0.001$ .



**NSAIDs**

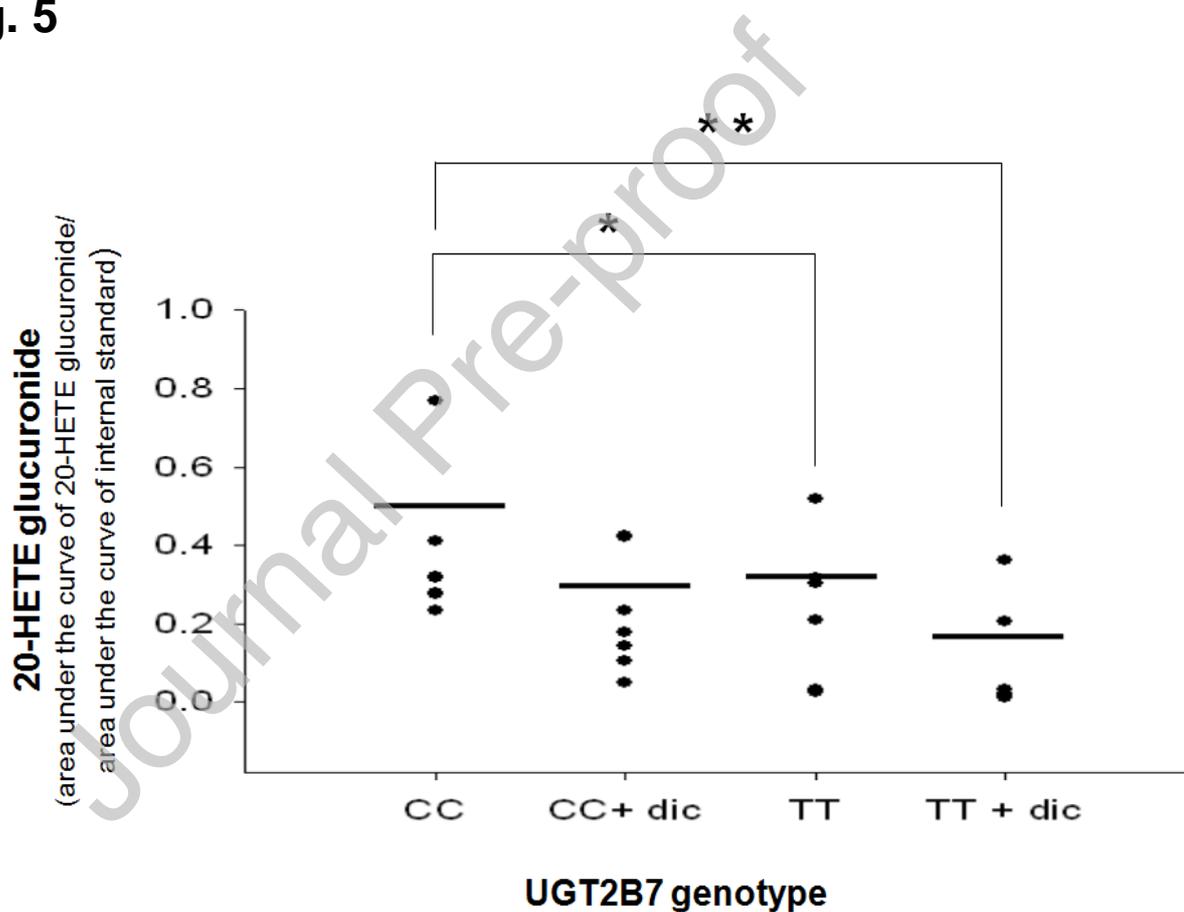
**Fig. 4.** Lineweaver-Burk plot of a Michaelis-Menten equation in the presence of diclofenac. Diclofenac showed a competitive inhibition mechanism with the  $K_m$  value of 20-HETE glucuronidation increasing from 23.5 to 66.7  $\mu\text{M}$  in the presence of 3.5  $\mu\text{M}$  diclofenac. The data are the mean of duplicate determination from a representative experiment.

**Fig. 4**



**Fig. 5.** Effects of diclofenac on 20-HETE glucuronidation in the presence and absence of the UGT2B7\*2 allele in HLMs. Inhibition of 20-HETE glucuronidation by diclofenac was further enhanced in UGT2B7\*2 HLMs compared with the wild-type genotype HLMs (\*\*,  $P < 0.01$ ). The genotype CC indicates wild-type UGT2B7 and TT indicates the homozygote UGT2B7\*2 genotype. “Dic” refers to diclofenac treatment. Statistical significance is indicated as \* $P < 0.05$ .

**Fig. 5**



**Table 1.** The IC<sub>50</sub> values against 20-HETE glucuronidation by NSAIDs

NSAID	Estimated IC <sub>50</sub> (μM)	
Diclofenac	3.5 ± 0.4 *	
Aspirin	ND	
Mefenamic acid	190 ± 23 *	
Meloxicam	ND	T
Celecoxib	77 ± 9 *	he
Indomethacin	220 ± 19 *	IC <sub>5</sub>
Ibuprofen	208 ± 20 *	o
Naproxen	91 ± 11 *	wa
Rofecoxib	ND	s
Probenecid	222 ± 23 *	cal
		cul

ated after incubation of pooled human liver microsomes with 10 μM of 20-HETE and seven different concentrations from each NSAID ranged from 0.1 to 1000 μM. The IC<sub>50</sub> was presented in the mean ± SD. ND, The IC<sub>50</sub> was not determined, since there was no inhibition activity of the NSAID against 20-HETE glucuronidation in the tested doses. \*, P<0.05 compared with the control probenecid.

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