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# Carboxyl-glucuronidation of mitiglinide by human UDP-glucuronosyltransferases

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ABSTRACT

Mitiglinide (MGN) is a new potassium channel antagonist for the treatment of type 2 diabetes mellitus. In the present study, a potential metabolic pathway of MGN, via carboxyl-linked glucuronic acid conjugation, was found. MGN carboxyl-glucuronide was isolated from a reaction mixture consisting of MGN and human liver microsomes fortified with UDP-glucuronic acid (UDPGA) and identified by a hydrolysis reaction with β-glucuronidase and HPLC-MS/MS. Kinetic analysis indicated that MGN from four species had the highest affinity for the rabbit liver microsomal enzyme ($K_m$ = 0.202 mM) and the lowest affinity for the dog liver microsomal enzyme ($K_m$ = 1.164 mM). The metabolic activity ($V_{max}/K_m$) of MGN to the carboxyl-glucuronidation was in the following order: rabbit > dog > rat > human. With the assessment of MGN glucuronide formation across a panel of recombinant UDP-glucuronosyltransferase (UGT) isoforms (UGT1A3, UGT1A4, UGT1A6, UGT1A9, and UGT2B7), only UGT1A3 and UGT2B7 exhibited high MGN glucuronosyltransferase activity. The $K_m$ values of MGN glucuronidation in recombinant UGT1A3 and UGT2B7 microsomes were close to those in human liver microsomes. The formation of MGN glucuronidation by human liver microsomes was effectively inhibited by quercetin (substrate for UGT1A3) and diclofenac (substrate for UGT2B7), respectively. The MGN glucuronidation activities in 15 human liver microsomes were significantly correlated with quercetin ($r^2$ = 0.806) and diclofenac glucuronidation activities ($r^2$ = 0.704), respectively. These results demonstrate that UGT1A3 and UGT2B7 are catalytic enzymes in MGN carboxyl-glucuronidation in human liver.



## 1. Introduction

Glucuronidation, catalyzed by UDP-glucuronosyltransferase (UGT), is one of the most common phase II biotransformations for a large number of therapeutic drugs. The UGTs are not only involved in the metabolism of many drugs but are also capable of the biotransformation of endogenous substrates and several xenobiotics [1,2]. UGTs are classified into either of two families, UGT1 or UGT2, based on their evolutionary divergence [3]. To date, nucleotide sequences encoding 18

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*Abbreviations:* ESI-MS/MS, electrospray ionization-tandem mass spectrometry; 7-HFC, 7-hydroxy-4-trifluoromethylcoumarin; HPLC-PDAD, high-performance liquid chromatography with photo-diode array detector; MGN, mitiglinide; UDPGA, UDP-glucuronic acid; UGT, UDP-glucuronosyltransferase

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human UGT proteins have been identified [4]. UGT catalyzes the transfer of the glucuronosyl moiety from the uridine 5′-diphosphoglucuronic acid (UDPGA) to a suitable functional group, such as an amino, carboxyl, hydroxyl, or sulfhydryl group, as well as an acidic carbon on a lipophilic substrate [4]. As a result of a hydrophilic glucuronosyl moiety being introduced into a drug molecule, glucuronides are generally less toxic, polar compounds and are readily excreted into the bile and/or urine. The determination of the identity of the enzymes involved in drug metabolism is important to understand interindividual variation in drug effect and metabolism. Moreover, the identification of the drug metabolizing enzymes (e.g., cytochrome P450 and UGT) could provide essential information about potential drug–drug interactions, since many enzymes are involved in the metabolism of several xenobiotics [5,6]. Enzyme kinetic parameters are also important for drug metabolism. A number of approaches have been developed for the identification of human UGTs on the glucuronidation of endogenous and exogenous compounds in vitro [7,8].

Mitiglinide (MGN), (−)-2(*S*)-benzyl-4-(*cis*-perhydroisoindol-2-yl) butyric acid, is a new potassium channel antagonist for the treatment of type 2 diabetes mellitus. It has potent oral hypoglycaemic activity and is structurally different from the sulphonylureas, although it stimulates calcium influx by binding to the suphonylurea receptor on pancreatic beta-cells and closing $K^+$-ATP channels. Its early insulin release and short duration of action would be effective in improving postprandial hyperglycemia [9]. In vitro and in vivo studies have demonstrated that the insulinotropic effect of MGN is more potent than that of nateglinide [10].

To date, metabolism information about MGN has not been reported. Therefore, the purpose of the present study was (i) to develop a simple HPLC-UV method for determining MGN glucuronides directly, (ii) to identify the human UGT isoform(s) responsible for the MGN glucuronidation, (iii) to determine the kinetics of MGN-glucuronide in human, dog, rabbit, rat liver microsomes and recombinant UGT microsomes from baculovirus-insect cells and (iv) furthermore, to confirm the contribution of the enzyme on MGN glucuronidation by inhibition analyses and correlation analyses with typical substrates for UGT isoforms.

## 2. Materials and methods

### 2.1. Materials

MGN calcium hydrate was kindly provided by Shangdong Chengchuang Medicine Co., Ltd. (Shangdong, China) with a purity of 99.8%. UDP-glucuronic acid (UDPGA), alamethicin, trifluoperazine, and β-D-glucuronidases from *Escherichia coli*, and 4-trifluoromethyl-7-hydroxycoumarin glucuronide were purchased from Sigma–Aldrich (St. Louis, MO). 7-Hydroxy-4-trifluoromethylcoumarin (7-HFC) was obtained from Acros Organics (Belgium). Propofol was purchased from ICN Biomedicals Inc. (USA). Bilirubin was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). All other reagents and solvents used were either of analytical or of HPLC grade.

### 2.2. Preparation of recombinant human UGTs

Recombinant human UGT (UGT1A3, UGT1A4, UGT1A6, UGT1A9 and UGT2B7) homogenates were prepared as described in our laboratory [11–13]. In brief, each of the *UGT* genes was excised from the pGEM-T vector, subcloned into the pFastBacl vector, and transformed into *E. coli* DH10Bac. PCR transposition of each *UGT*-specific primer and M13 primer was used to confirm the transposition of their genes into the bacmid. The minipreparation of each bacmid-*UGT* was transfected into Sf9 cells. After 72 h, the first passage of baculovirus was harvested from supernatant and titrated by viral plaque assay. Infection conditions were optimized for subsequent amplification or expression. Infected cells were collected by centrifugation and disrupted by sonication. The relative expression level of each UGT was ascertained by Western blot using the mouse anti-His antibody. A peroxidase-conjugated goat anti-mouse antibody was used as the secondary antibody. Protein concentration of the cell homogenate was determined using Lowry's method. Cell homogenate was stored frozen at approximately −80 °C until use. Transfection with an untransposed bacmid was performed as a negative control.

### 2.3. Preparation of liver microsomes

Human liver samples from 15 cancer patients (5 females, 10 males) were obtained from the Sir Run Run Shaw Hospital, Zhejiang, China. Informed consent was obtained from each patient prior to study entry. The present study was approved by an Ethics Committee of the Sir Run Run Shaw Hospital. All patients had undergone partial hepatectomy to remove liver cancer. Pathologically and histologically normal liver samples used in the study were obtained from normal portions of removed tissue. All of the fresh samples were rapidly frozen in liquid nitrogen and stored at −80 °C before use. Preparation of liver microsomes from humans ($n = 15$), male Sprague–Dawley rats, male rabbits and male beagle dogs ($n = 5$) was performed as published methods [14]. All microsomes were stored frozen at approximately −80 °C until use. The protein concentration of the microsomes was determined using Lowry's method.

### 2.4. MGN glucuronidation assay

A typical incubation mixture (100 μl total volume) contained 50 mM Tris–HCl buffer, pH 7.5, 5 mM $MgCl_2$, 5 mM UDP-glucuronic acid, 25 μg/ml alamethicin, 0.5 mg/ml human liver microsomes (recombinant UGTs, or experimental animal liver microsomes), and 100 μM MGN. MGN was dissolved in methanol. The final concentration of methanol in the reaction mixture was 1% (v/v). After preincubation at 37 °C for 5 min, the reaction was initiated by the addition of UDPGA. The reaction mixture was incubated at 37 °C for 30 min and the reaction was stopped with 100 μl of ice-cold acetonitrile. After removal of the protein by centrifugation at 9300 × *g* for 10 min, a portion of the sample was subjected to HPLC. Chromatography was performed using a Shimadzu HPLC system (model 2010C; Shimadzu, Japan), equipped with a UV detector. Separation was performed on a Diamonsil-C18 column (4.6 mm × 200 mm, 5 μm; Dikma Technologies, Dalian, China)

preceded by an ODS guard column (10 mm × 5 mm, 5 μm) at 20 °C. Analysis was isocratic at 1.0 ml/min flow rate with acetonitrile/0.02 M $KH_2PO_4$ buffer (pH 4.0) (33:67, v/v) as the mobile phase. The eluate was monitored at 210 nm. The retention times of the MGN glucuronide and MGN were 4.5 and 16.8 min, respectively.

Due to the absence of authentic standards for MGN glucuronide, quantification of the glucuronide in the incubation mixtures was accomplished using standard curves for MGN. In addition, for the quantification of MGN glucuronide, the eluate of the HPLC from the incubation mixture with human liver microsomes was collected. A part of the eluate was hydrolyzed with β-glucuronidase, as described below. The completely hydrolyzed MGN glucuronide was quantified as MGN by HPLC. We determined the peak area at 210 nm and calculated its apparent factor according to the known contents of MGN glucuronide. The factor was applied to the calculation of MGN glucuronide formed in the incubation mixture.

### 2.5. Hydrolysis with β-glucuronidase

The eluate corresponding to MGN glucuronide was evaporated in vacuo and reconstituted in 1.0 ml of 0.05 M sodium acetate buffer (pH 5.0). Each sample was incubated in the absence (control) and presence of 100 units of β-glucuronidase at 37 °C for 24 h. Aliquots (100 μl) of each incubation were stopped with 100 μl of ice-cold acetonitrile. After removal of the protein by centrifugation, the supernatant was subjected to HPLC as described above.

### 2.6. Identification of MGN glucuronide by HPLC–ESI-MS/MS analysis

The incubation of MGN with human liver microsomes for structure identification was carried out as described above. Detection of MGN glucuronide was achieved with injection of 20 μl of the centrifugal supernatant onto the HPLC-MS/MS system. HPLC-MS/MS analysis was performed using a Finnigan LCQ/DECA XP (Thermo) mass spectrometer coupled to an Agilent model 1100 series HPLC system (Agilent Technologies, Palo Alto, CA). Ionization of the analytes was achieved by electrospray in the positive ion mode. The same analysis column as above was used for the HPLC separation. The HPLC separation was carried out at 20 °C using a gradient composed of mobile phase A (0.01 M ammonium formate buffer solution, pH 4.0) and mobile phase B (acetonitrile). The gradient, expressed as changes in mobile phase B, was as follows: 0–8 min, hold at 40% B; 8–10 min, a linear increase from 40% to 80% B; 10–18 min, hold at 80% B. The mobile phase flow rate was 0.6 ml/min with 1:6 split. MS conditions used were as follows: capillary voltage, 4.5 kV; cone voltage, 12 V; cone gas, 10 arb; desolvation gas, 30 arb; desolvation temperature, 350 °C; source temperature, 100 °C. MS/MS spectra were obtained in the range of *m*/z 50–800.

### 2.7. Kinetic analyses

The kinetics studies were performed using the liver microsomes obtained from human, rat, dog, and rabbit ($n = 5$), and recombinant UGT1A3 and UGT2B7 expressed in the homogenate. In determining the kinetic parameters, the MGN concentration ranged from 20 to 2000 μM. The concentration of microsomal protein and incubation time were 1 mg/ml and 30 min, respectively, except for the case of rabbit liver microsomes, which was 15 min. Concentrations of UDP-glucuronic acid in the incubation mixtures were 5 mM. Kinetic parameters were estimated from the fitted curves using the Prism computer program (GraphPad Software Inc., San Diego, CA), designed for nonlinear regression analysis. The following equation was applied, assuming a Michaelis–Menten equation: $V = V_{max} \times [S] / (K_m + [S])$, where $V$ is the rate of reaction, $V_{max}$ the maximum velocity, $K_m$ the Michaelis constant (substrate concentration at $0.5V_{max}$), and [S] is the substrate concentration.

### 2.8. Determination of enzyme activity of recombinant UGTs and microsomes

The glucuronosyltransferase activities of recombinant UGTs (UGT1A3, UGT1A4, UGT1A6, UGT1A9, UGT2B7) and human liver microsomes ($n = 5$) were determined in assays using HPLC and 7-HFC as the substrate. An incubation mixture (100-μl total volume) contained 50 mM Tris–HCl buffer, pH 7.5, 8 mM $MgCl_2$, 2 mM UDPGA, 25 μg/ml alamethicin, 0.5 mg/ml microsomes, and 100 μM 7-HFC. 7-HFC was dissolved in methanol. The final concentration of methanol in the reaction mixture was 1% (v/v). After preincubation at 37 °C for 5 min, the reaction was initiated by the addition of UDPGA. The reaction mixture was incubated at 37 °C for 30 min and the reaction was stopped with 100 μl of ice-cold acetonitrile. After removal of the protein by centrifugation at 9300 × *g* for 10 min, a 50 μl portion of the sample was subjected to HPLC. Chromatography was performed using a Shimadzu 2010C HPLC system and a Diamonsil-C18 column (4.6 mm × 200 mm, 5 μm; Dikma Technologies, Dalian, China). The column temperature was 25 °C and the eluate was monitored at 325 nm. Mobile phases A and B were water and acetonitrile, respectively. A linear gradient of 10–65% mobile phase B over 10 min was used for the analysis of 7-HFC and its glucuronide, at a flow rate of 1.0 ml/min. The retention times of 7-HFC and its glucuronide were 12.3 and 10 min, respectively. The formation of glucuronide was quantified by comparing the peak areas in the incubations to a standard curve for 4-trifluoromethyl-7-hydroxycoumarin glucuronide. Recombinant UGT1A4 microsomes do not form glucuronides with 7-HFC as a substrate. Therefore, the activity of UGT1A4 was determined in assays using HPLC and trifluoperazine as a substrate [15].

### 2.9. Correlation analysis

The glucuronidation activity of MGN at a concentration of 100 μM was measured in a bank of human liver microsomes from 15 individual donors (12 individual donors liver microsomes for correlation analysis between the glucuronidation activity of MGN and the glucuronidation activity of bilirubin) and then compared with UGT-selective marker activities (the final concentration also 100 μM). Pearson's product-moment correlation coefficient ($r$) was used to assess the relationship between glucuronidation activities for MGN and UGT1A1-selective bilirubin glucuronidation activity [16,17], UGT1A3-selective quercetin glucuronidation activity [12], UGT1A4-selective

trifluoperazine glucuronidation activity [1], UGT1A9-selective propofol glucuronidation activity [18], and diclofenac for UGT2B7 [19,20]. The computer program SPSS 11.5 (SPSS Inc., Chicago, USA) was used to conduct the correlation analysis.

### 2.10. *Inhibition analysis of MGN glucuronosyltransferase activity in humanliver microsomes*

The glucuronidation activity of MGN at a concentration of 350 μM was measured in pooled human liver microsomes in the presence of known UGT inhibitors. The chemical inhibitors include bilirubin for UGT1A1 [16,17], quercetin for UGT1A3 [13], trifluoperazine for UGT1A4 [21], propofol for UGT1A9 [22,23], and diclofenac for UGT2B7 [19,20]. All inhibitors were dissolved in dimethyl sulfoxide and were added to the reaction mixtures at final concentrations ranging from 1 to 300 μM. Control incubations containing all components of the reaction mixtures, including 1% (v/v) dimethyl sulfoxide (5% for bilirubin), but not the inhibitors, were performed in parallel. The glucuronidation activities were calculated as a percentage of control activity, and the $IC_{50}$ values were calculated from the fitted curves using the SPSS computer program (SPSS Inc., Chicago, USA), designed for nonlinear regression analysis.

## 3. Results

### 3.1. *Identification of MGN glucuronide formed by human liver microsomes*

Enzymatic formation of MGN glucuronide by human liver microsomes was first characterized by HPLC (Fig. 1A and B) and HPLC–ESI-MS/MS (Fig. 2). Fig. 1B shows representative HPLC chromatograms of enzymatically formed MGN glucuronide by human microsomes in the presence of UDPGA. The MGN glucuronide formed in the reaction mixture had a retention time of 5.4 min. The mass spectrum showed an [M + H] + ion at *m*/*z* 492.38, corresponding to MGN glucuronide and a fragment ion at *m*/*z* 316.46, corresponding to the parent drug MGN + 2H with loss of the glucuronic acid moiety (176 amu). The same metabolite, MGN glucuronide, was formed in humans and experimental animal liver microsomes such as rat, rabbit and dog (figures not shown).

### 3.2. *Hydrolysis with β-glucuronidase*

MGN glucuronide was easily hydrolyzed with β-glucuronidase and converted to parent MGN (Fig. 1C and D). No change was observed in the glucuronide over the 20 h of incubation without β-glucuronidase. At the same time, change was also not observed when MGN glucuronide was placed for 24 h at 4 °C. Therefore, all detected MGN in the presence of the β-glucuronidase can be attributed to enzymatic hydrolysis. The apparent factor calculated from the peak areas over the known contents of MGN glucuronide was 0.97. Thus, the factor was applied to the quantification of MGN glucuronide formed in the microsomal incubation mixture.

### 3.3. *Kinetics of MGN glucuronidation in liver microsomes*

Kinetic analysis of MGN glucuronidation was performed in liver microsomes obtained from humans and experimental animals ($n = 5$). As shown in Fig. 3, the MGN glucuronidation by human liver microsomes displayed typical Michaelis–Menten

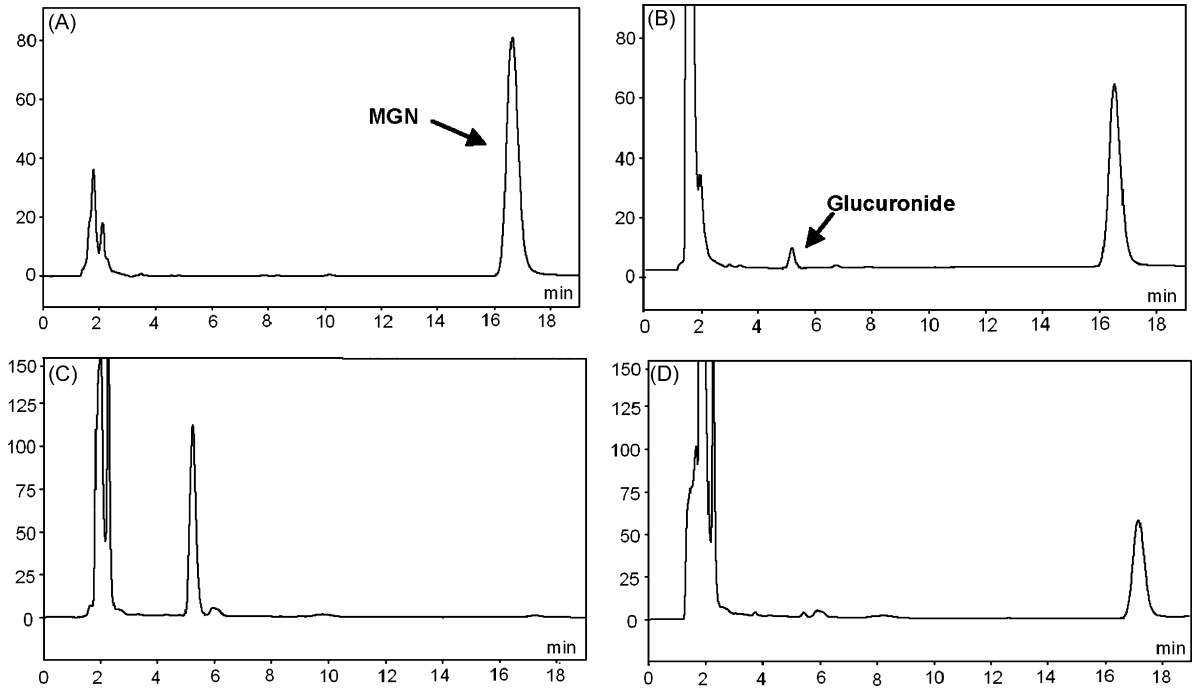


**Fig. 1 – Representative HPLC chromatograms of the formation of MGN glucuronide in human liver microsomes and hydrolyzing of MGN glucuronide by β-D-glucuronidase. (A) Incubate mixtures without UDPGA; (B) human liver microsomes (1.0 mg/ml protein) was incubated at 37 °C for 1 h with 100 μM MGN in the presence of 5 mM UDPGA; (C) collected MGN glucuronide (about 50 μM); (D) MGN glucuronide was hydrolyzed by β-D-glucuronidase at 37 °C for 24 h.**

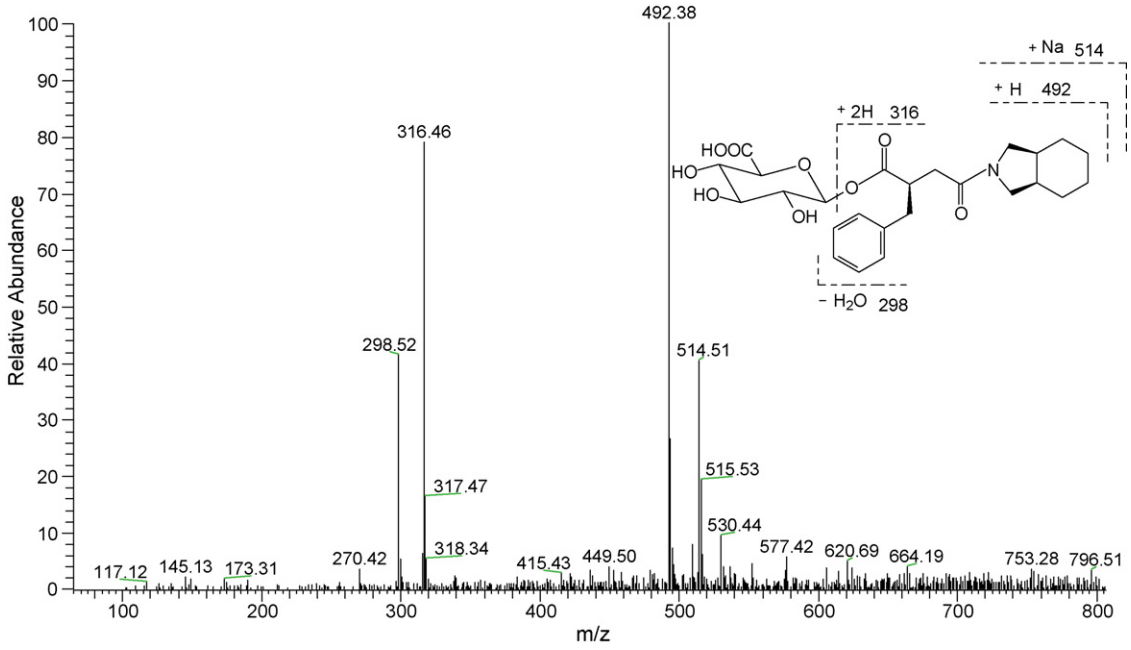


**Fig. 2 – Representative mass spectrum of MGN glucuronide formed from MGN by human liver microsomes. The spectrum was taken at the retention time of 5.4 min in Fig. 1B.**

kinetics. Additionally, an Eadie–Hofstee plot was monophasic. Fitting the data points to the Michaelis–Menten equation yielded the kinetic parameters listed in Table 1. Incubation of various concentrations of MGN with human liver microsomes demonstrated that the $K_m$, $V_{max}$, and $CL_{int}(V_{max}/K_m)$ values for MGN glucuronide were 0.329 ± 0.029 mM, 0.066 ± 0.002 nmol/(min mg(protein)), and 0.201 μl/(min mg(protein)), respectively. On the other hand, incubation of various concentrations of MGN with dog, rabbit and rat liver microsomes demonstrated that the $CL_{int}$ values for MGN glucuronide were 0.664, 1.152 and 0.570 μl/(min mg(protein)), respectively.

### 3.4. *Activities of recombinant UGTs and human liver (positive control)*

The results of the activity determination of five recombinant UGT isoforms expressed in baculovirus-infected insect cells, and human liver microsomes are presented in Table 2. These results served as positive controls for the glucuronosyltransferase activities and demonstrated that the recombinant UGTs and microsomes were active.

### 3.5. *MGN glucuronidation in recombinant UGT isoforms*

All recombinant UGTs were used to determine their MGN glucuronosyltransferase activities (Fig. 4). Only UGT1A3 and UGT2B7 exhibited remarkably high MGN glucuronidation activities. All other isoforms (UGT1A4, UGT1A6, and UGT1A9) had very low MGN glucuronidation activities or no activities. Kinetics analyses of the MGN glucuronidation in recombinant UGT1A3 and UGT2B7 were performed (Fig. 3B and C). MGN glucuronidation by recombinant UGT1A3 and UGT2B7 also displayed typical Michaelis–Menten kinetics, similar to that by human liver microsomes. Additionally, each Eadie–Hofstee plot was monophasic. Fitting the data points to the Michaelis–Menten equation yielded the kinetic parameters listed in Table 1. Incubation of various concentrations of MGN with recombinant UGT1A3 and UGT2B7 demonstrated that the $K_m$,

**Table 1 – Kinetic parameters of MGN glucuronidation in microsomes obtained from humans and experimental animals, and recombinant UGT1A3 and UGT2B7 ($n$ = 5)**

| Source of UGT | $K_m$ (mM) | $V_{max}$ (nmol/(min mg(protein))) | $V_{max}/K_m$ (μl/(min mg(protein))) |
|---|---|---|---|
| Human liver | 0.329 ± 0.029 | 0.066 ± 0.002 | 0.201 |
| Dog liver | 1.164 ± 0.008 | 0.773 ± 0.080 | 0.664 |
| Rabbit liver | 0.210 ± 0.031 | 0.242 ± 0.082 | 1.152 |
| Rat liver | 0.598 ± 0.043 | 0.341 ± 0.042 | 0.570 |
| UGT1A3 | 0.455 ± 0.029 | 0.028 ± 0.001 | 0.062 |
| UGT2B7 | 0.447 ± 0.023 | 0.019 ± 0.000 | 0.043 |

MGN was incubated with microsomes and UDP-glucuronic acid for 30 min (20 min in the case of rabbit liver microsomes and 1 h in the case of recombinant UGT1A3 and UGT2B7). The kinetic parameters were calculated with GraphPad Prism software. Each value represents mean ± S.E. of triplicate points.

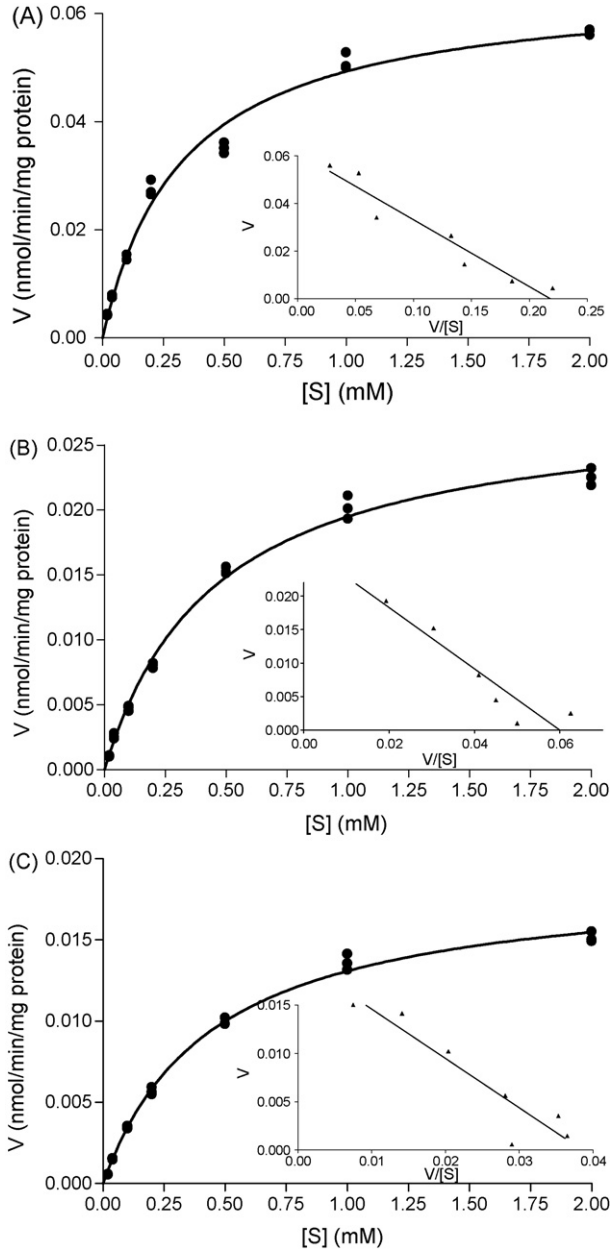


**Fig. 3 – Kinetics of MGN glucuronidation in human liver (A), recombinant UGT1A3 (B), and recombinant UGT2B7 (C) microsomes. The concentration of MGN ranged from 20 to 2000 μM. The formation of MGN glucuronide was determined as described in Section 2. Each inset shows the Eadie–Hofstee plot of the experimental data. Each incubation was performed by triplicate determinations.**

$V_{max}$, and $CL_{int}$ values for MGN glucuronide were 0.455 ± 0.029 and 0.447 ± 0.023 mM, 0.028 ± 0.001 and 0.019 ± 0.000 nmol/(min mg(protein)), and 0.062 and 0.043 μl/(min mg(protein)), respectively.

### 3.6. Correlation study

Correlation analyses were performed between the MGN glucuronosyltransferase activity and bilirubin (UGT1A1), quercetin (UGT1A3), trifluoperazine (UGT1A4), propofol (UGT1A9), or diclofenac (UGT2B7) glucuronosyltransferase activities in 15 (12 for bilirubin) human liver microsomes. The MGN glucuronosyltransferase activities were significantly correlated with the quercetin glucuronosyltransferase activities ($r^2 = 0.806$, $P < 0.001$) and diclofenac glucuronosyltransferase activities ($r^2 = 0.704$, $P < 0.001$) (Fig. 5B and C), although the MGN glucuronosyltransferase activities did not correlate with the propofol ($r^2 = 0.074$), trifluoperazine ($r^2 = 0.485$) and bilirubin ($r^2 = 0.0008$) glucuronosyltransferase activities (Fig. 5A, D and E).

**Table 2 – Determination of activities of recombinant UGTs and human liver microsomes ($n = 5$)**

| Source of UGT | Probe substrate | UGT activity (pmol/(min mg(protein))) |
|---|---|---|
| UGT1A3 | 7-HFC | 52.5 ± 7.6 |
| UGT1A4 | Trifluoperazine | 233.3 ± 20.3 |
| UGT1A6 | 7-HFC | 38.0 ± 5.3 |
| UGT1A9 | 7-HFC | 203.4 ± 19.2 |
| UGT2B7 | 7-HFC | 20.6 ± 3.2 |
| Insect control | 7-HFC | N.D. |
| Human liver | 7-HFC | 624.2 ± 42.3 |

Each value represents mean ± S.E. of triplicate points.

### 3.7. Inhibition analyses of MGN glucuronidation in human liver microsomes

The inhibitory effects of bilirubin (UGT1A1), quercetin (UGT1A3), trifluoperazine (UGT1A4), propofol (UGT1A9), and diclofenac (UGT2B7) on the MGN glucuronosyltransferase activities in human liver microsomes were investigated. As shown in Fig. 6, the MGN glucuronosyltransferase activity in human liver was prominently inhibited by quercetin and diclofenac ($IC_{50}$ = 28.9 ± 3.76 and 9.9 ± 1.8 μM, respectively). Propofol, trifluoperazine and bilirubin showed weak inhibitory effects ($IC_{50} > 100$ μM).

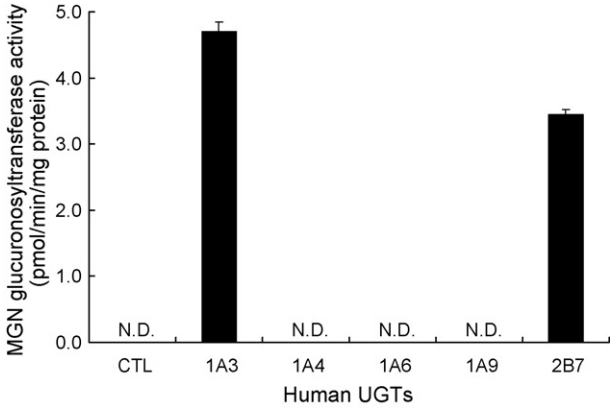


**Fig. 4 – MGN glucuronosyltransferase activity in recombinant human UGTs. The formation of MGN glucuronide in recombinant UGTs was determined at 100 μM MGN. Each column represents the mean ± S.E. of triplicate determinations. The lower limit of determination of the assay under these conditions was 5 μmol $l^{-1}$.**

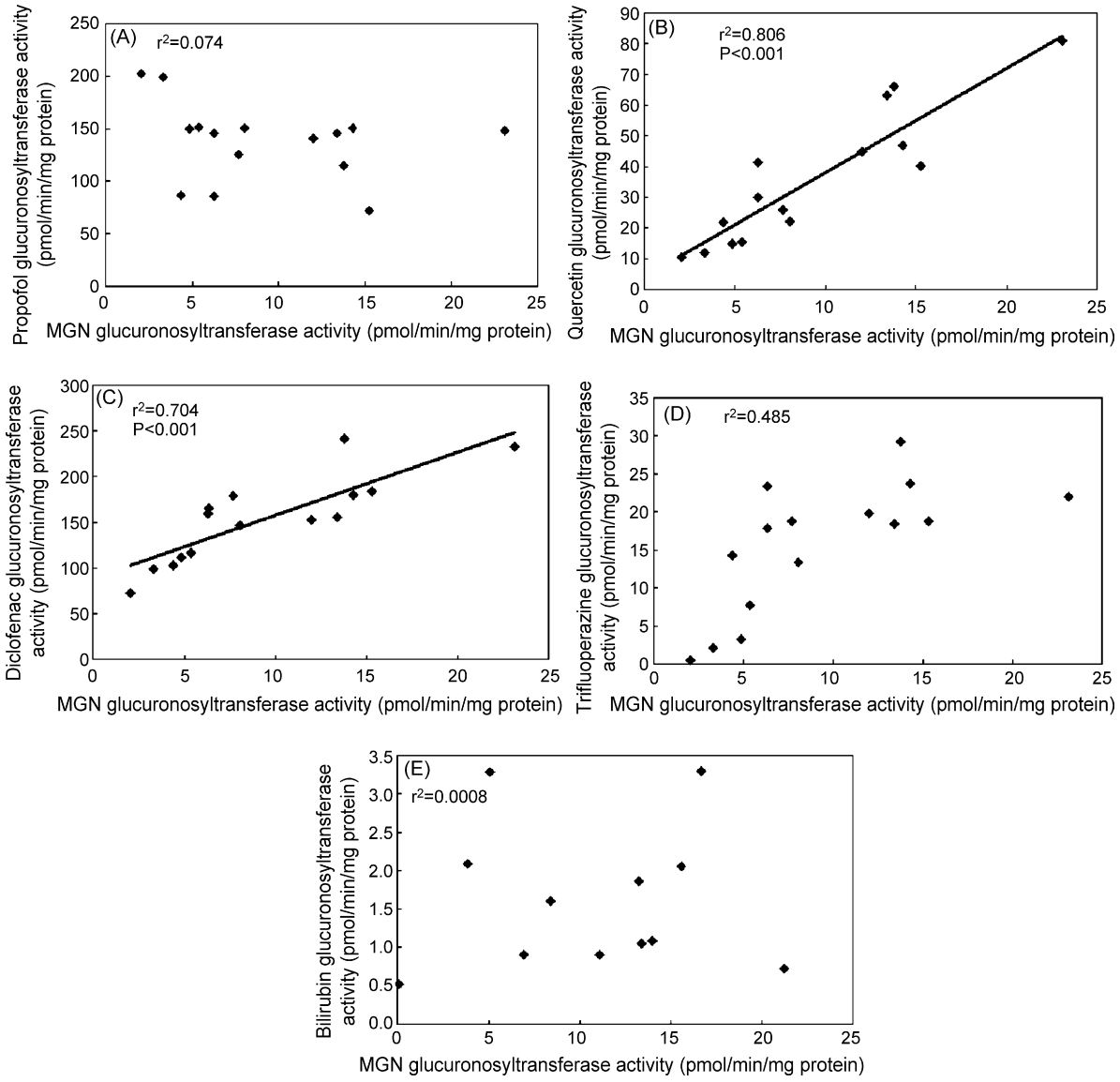


**Fig. 5 – Correlation between MGN glucuronosyltransferase activity and other glucuronosyltransferase activities in microsomes from 15 (12 for the correlation study between MGN glucuronosyltransferase activity and bilirubin glucuronosyltransferase activities) donors. MGN and UGT-selective marker glucuronosyltransferase activities in human microsomes were all determined at 100 μM. MGN glucuronidation is significantly related to that of quercetin ($r^2$ = 0.806, P < 0.001, B) and diclofenac ($r^2$ = 0.704, P < 0.001, C). No correlation was observed with propofol ($r^2$ = 0.074, A), or trifluoperazine ($r^2$ = 0.485, D), or bilirubin ($r^2$ = 0.0008, E).**

## 4. Discussion

Carboxyl-glucuronidation plays a significant role in the metabolic elimination of many carboxylic acid-containing therapeutic agents, such as non-steroidal anti-inflammatory drugs (NSAID) [24]. In the structure of MGN, there is also a carboxyl group, which can possibly be linked to glucuronic acid. The same metabolite, MGN carboxyl-glucuronide, identified by HPLC (Fig. 1) and LC-MS/MS (Fig. 2) analyses, was detected in dog, rat, rabbit and human liver microsomal incubations. Although MGN metabolite(s) have not been reported in vivo, the results from our in vitro studies suggested that glucuronidation was an elimination pathway for MGN. Enzyme kinetic parameters are important for drug metabolism. The kinetic analysis results indicated that MGN had the highest affinity for the rabbit liver microsomal enzyme ($K_m$ = 0.202 mM) and the lowest affinity for the dog liver mcirosomal enzyme ($K_m$ = 1.164 mM) in four species. The metabolic activity ($V_{max}/K_m$) of MGN to the carboxyl-glucuronidation was in the following order: rabbit > dog > rat > huhuman (Table 1). The results indicated that MGN was a poor UGT substrate in man compared to the three animal species. So we supposed that other metabolic pathways were probably involved in man. Then we tried to study whether MGN is metabolized by cytochrome P450 enzymes in vitro using human and rat liver microsomes by HPLC-PDAD. Dismayingly, no metabolites were found and MGN concentration in microsomal incubates did not decrease (data were not shown).

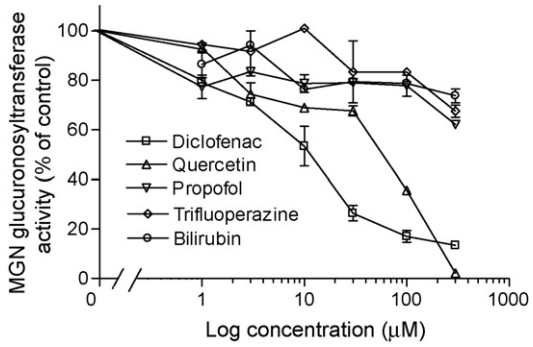


**Fig. 6 – Inhibitory effects of UGTs inhibitors on MGN glucuronosyltransferase activity in human liver microsomes. MGN glucuronosyltransferase activities at 350 μM MGN in human liver microsomes were determined in the presence or absence of UGTs inhibitors as described in Section 2. Each value represents the mean of duplicate incubations. The $IC_{50}$ values were 9.9 μM for diclofenac and 28.9 μM for quercetin. Propofol, trifluoperazine and bilirubin showed weakly inhibit effect ($IC_{50}$ > 100 μM).**

In these studies, the microsomal protein concentration was 1 mg/ml and the incubation time was 30 min. The other incubation conditions referred to Yu et al. [25]. Certainly, it should be noted that a UV detector was used in these studies. So it is possible that some metabolites of MGN may be not detected. Except for UGT and CYP mediated metabolic pathways, there are other pathways in the metabolism of carboxylic acid-containing drugs, such as aminoacid conjugation, which should be researched in future studies.

At least 17 UGT mRNAs are known to exist in the human. These are divided into two families, UGT1 and UGT2, consisting of nine and eight isoforms, respectively, on the basis of amino acid sequence identity. Among these isoforms, five isoforms of the UGT1A subfamily, UGT1A1 [26], UGT1A3 [27], UGT1A4 [26], UGT1A6 [28], and UGT1A9 [29], and seven isoforms of the UGT2B subfamily, UGT2B4 [30], UGT2B7 [31], UGT2B10 [30], UGT2B11 [32], UGT2B15 [33], UGT2B17 [34,35], and UGT2B28 [36], are known to be expressed in the human liver. To our knowledge, xenobiotics that have carboxyl groups are often used as potential substrates of the recombinant human UGT1A3, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10 and UGT2B7 [37–39]. UGT1A7, 1A8 and 1A10, however, apparently occur only in the gastrointestinal tract [4]. To identify the human UGT isoform(s) responsible for the formation of the carboxyl glucuronide of MGN, we first examined the activities and kinetics in recombinant UGT microsomes from baculovirus-insect cells. Five isoforms of the UGT subfamily, UGT1A3, 1A4, 1A6, 1A9 and 2B7, were used, which are currently available in our lab. It should be noted that we have no evidence of a role in MGN carboxyl-glucuronidation by the other human hepatic UGTs. Consequently, UGT1A3 and UGT2B7 exhibited high MGN glucuronosyltransferase activity among the UGT isoforms examined. Furthermore, it was confirmed that the $K_m$ values of MGN glucuronidation in recombinant UGT1A3 and UGT2B7 were similar to those in human liver microsomes (Table 1). To clarify the contribution of UGT1A3 and UGT2B7 for MGN glucuronidation, inhibition studies and correlation analyses with typical substrates for UGT isoforms were performed using human liver microsomes. Quercetin, a substrate for UGT1A3 [12], inhibited the MGN glucuronosyltransferase activity in human liver microsomes with an $IC_{50}$ value of 28.9 μM (Fig. 6). The MGN glucuronosyltransferase activities in 15 human liver microsomes were significantly correlated with the quercetin glucuronosyltransferase activities ($r^2 = 0.806$). At the same time, diclofenac, a substrate for UGT2B7 [19,20], also inhibited the MGN glucuronosyltransferase activity in human liver microsomes with an $IC_{50}$ value of 9.9 μM. The relation between MGN and diclofenac glucuronosyltransferase activities was also significantly correlated ($r^2 = 0.704$). These results suggested that UGT1A3 and UGT2B7 are the major UGT isoforms catalyzing MGN glucuronidation in human liver. Although UGT1A1 is currently not available in our lab, it is an important isoenzyme in UGTs. In the present study, bilirubin, a selective substrate of UGT1A1 [16,17], was used in a correlation and inhibition study. Both results indicated that MGN was not a substrate of UGT1A1. But it should be further corroborated using recombinant UGT1A1.

Repaglinide, nateglinide and mitiglinide are three effective drugs in mediating the release of insulin from beta-cells. Repaglinide is metabolized predominantly in the liver by the cytochrome P450 enzymes, with the principal isoforms involved being CYP2C8 and CYP3A4 [40,41]. Repaglinide glucuronide was detected, but the content was very low [42]. In vitro data demonstrated that nateglinide is predominantly metabolized by cytochrome P450 isoenzymes CYP2C9 (70%) and CYP3A4 (30%) [43]. A glucuronic acid conjugate resulting from direct glucuronidation of the carboxylic acid of nateglinide was also present, but as in the case of repaglinde, the content was also small [44]. To date, information regarding in vivo or in vitro MGN metabolism has not been reported. The present results indicate the metabolism of MGN is different compared to that of repaglinide and nateglinide.

In summary, our data indicates that UGT1A3 and UGT2B7 are important catalytic enzymes in MGN carboxyl-glucuronidation in human liver.

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