Research Article

Regioselective Glucuronidation of Andrographolide and Its Major Derivatives: Metabolite Identification, Isozyme Contribution, and Species Differences

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Abstract. Andrographolide (AND) and two of its derivatives, deoxyandrographolide (DEO) and dehydroandrographolide (DEH), are widely used in clinical practice as anti-inflammatory agents. However, UDP-glucuronosyltransferase (UGT)-mediated phase II metabolism of these compounds is not fully understood. In this study, glucuronidation of AND, DEO, and DEH was characterized using liver microsomes and recombinant UGT enzymes. We isolated six glucuronides and identified them using 1D and 2D nuclear magnetic resonance (NMR) spectroscopy. We also systematically analyzed various kinetic parameters (K_m , V_{max} , and CL_{int}) for glucuronidation of AND, DEO, and DEH. Among 12 commercially available UGT enzymes, UGT1A3, 1A4, 2B4, and 2B7 exhibited metabolic activities toward AND, DEO, and DEH. Further, UGT2B7 made the greatest contribution to glucuronidation of all three anti-inflammatory agents. Regioselective glucuronidation showed considerable species differences. 19-*O*-Glucuronides were present in liver microsomes from all species except rats. 3-*O*-Glucuronides were produced by pig and cynomolgus monkey liver microsomes for all compounds, and 3-*O*-glucuronide of DEH was detected in mouse and rat liver microsomes (RLM). Variations in K_m values were 48.6-fold (1.93–93.6 μ M) and 49.5-fold (2.01–99.1 μ M) for 19-*O*-glucuronide and 3-*O*-glucuronide formation, respectively. Total intrinsic clearances (CL_{int}) for 3-*O*- and 19-*O*-glucuronidation varied 4.8-fold (22.7–110 μ L min⁻¹ mg⁻¹), 10.6-fold (94.2–991 μ L min⁻¹ mg⁻¹), and 8.3-fold (122–1,010 μ L min⁻¹ mg⁻¹), for AND, DEH, and DEO, respectively. Our results indicate that UGT2B7 is the major UGT enzyme involved in the metabolism of AND, DEO, and DEH. Metabolic pathways in the glucuronidation of AND, DEO, and DEH showed considerable species differences.

KEY WORDS: andrographolide; anti-inflammatory activity; glucuronidation; species difference; UGT2B7.

Xiangge Tian, Sicheng Liang, and Chao Wang contributed equally to this work.

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ABBREVIATIONS: AND, Andrographolide; DEH, Dehydroandrographolide; DEO, Deoxyandrographolide; HLM, Human liver microsomes; PLM, Pig liver microsomes; RLM, SD rat liver microsomes; MLM, Mouse liver microsomes; DLM, Dog liver microsomes; CyLM, Monkey liver microsomes; UDPGA, Uridine diphosphate glucuronic acid; UGTs, UDP-glucuronosyltransferases; AND-1, Andrographolide-19-*O*- β -D-glucuronide; AND-2, Andrographolide-3-*O*- β -D-glucuronide; DEH-1, Dehydroandrographolide-19-*O*- β -D-glucuronide; DEH-2, Dehydroandrographolide-3-*O*- β -D-glucuronide; DEO-1, Deoxyandrographolide-19-*O*- β -D-glucuronide; DEO-2, Deoxyandrographolide-3-*O*- β -D-glucuronide.

INTRODUCTION

Andrographolide (AND), deoxyandrographolide (DEO), and dehydroandrographolide (DEH) (Fig. 1) are used extensively as anti-inflammatory agents. They are ent-labdane diterpenoids (1) from the herb *Andrographis paniculata* (Burm. f.) Nees. These compounds are well known for their beneficial pharmacological effects, which include anti-inflammatory (2,3), antiplatelet aggregation (4), antihyperglycemic (5), choleric (6), antitumor (7), and antihuman immunodeficiency virus effects (8). Recent studies have shown that the anti-inflammatory activity of AND is mediated predominately by inhibition of nitric oxide (NO) and PGE2 production, while antitumor activity is modulated by NF- κ B and MMP-9 signaling pathways (3). In many Asian countries including China, India, and Thailand, AND has been developed as an oral drug for treating various diseases, including respiratory infection, bacterial dysentery, and fever. Its compound preparations (Chuanxinlian tablet) containing AND, DEO, and DEH is available over the counter in China.

Although AND, DEO, and DEH are widely used in clinical practice, they exhibit poor bioavailability in humans

Fig. 1. Structures of andrographolide, dehydroandrographolide, deoxyandrographolide, and their glucuronides

after oral administration due to extensive first-pass metabolism and low water solubility (9). In previous studies (10), the primary metabolic profiles of AND in pooled human liver microsomes (HLMs), dog liver microsomes (DLMs), and rat liver microsomes (RLMs) were identified by LC-MS/MS. Metabolic differences between CYP450 enzymes from human, rat, and dog along with an NADPH-generating systems were measured using a semiquantitative method. Notably, only glucuronide and sulfate metabolites of AND were detected in human urine after oral intake of AND. AND-19-*O*- β -D-glucuronide accounts for over 80% of total metabolites in human urine (11–14). This evidence strongly indicates that glucuronidation is important in human metabolism of AND. However, the glucuronidation pathways of AND and its derivatives, mediated by UDP-glucuronosyltransferases (UGTs), have not been fully elucidated.

UGTs are versatile membrane-bound enzymes that play an important role in the elimination of endogenous compounds (e.g., steroids and thyroid hormones, fatty acids, bile acids, and bilirubin) and xenobiotics (e.g., drugs and environmental compounds) (15). Approximately 40–70% of all drugs cleared by phase II enzymes in humans are eliminated *via* UGT-mediated conjugation (16–20). At least 45 UGT isozymes are known in mammals. These UGTs can be classified into two families (UGT1 and UGT2) based on their sequence identities (19). Additionally, human and animal species have a different UGT gene system. For example, human UGT1A9 is functional, but rat UGT1A9 is a pseudogene (21). This may lead to significant differences in glucuronidation between different species, thereby adding a complexity in the extrapolation of animal data to humans. A variety of factors such as gene polymorphism, age, and even coadministered drugs can greatly influence glucuronidation. Identification of the enzymes involved in drug metabolism is therefore critical to understanding of how a drug acts in humans of different ethnicities and characteristics.

Our objectives of the present study were (1) to characterize *in vitro* glucuronide of AND, DEH, and DEO; (2) to identify the key human UGT enzymes responsible for glucuronidation of these three compounds; (3) to determine the enzyme kinetics for glucuronidation of AND, DEH, and DEO using recombinant human UGTs and various liver microsomes from human (HLM), monkey (CyLM), dog (DLM), pig (PLM), rat (RLM), and mouse (MLM).

MATERIALS AND METHODS

Materials

UDPGA, β -estradiol-3-(β -D-glucuronide) sodium, azidothymidine (AZT), fluconazole, phenylbutazone, diclofenac, 20(*S*)-protopanaxatriol (PPT), *Escherichia coli* lipopolysaccharide (LPS), and antibiotics were purchased from Sigma-Aldrich (St. Louis, MO, USA). Rabbit antihuman UGT2B7 polyclonal antibody was purchased from BD Gentest (Woburn, MA). AND, DEO, and DEH were isolated from *A. paniculata* and identified by NMR and ESI mass spectrometry as described previously (22). Their purities were greater than 98% as determined by high-performance liquid chromatography with diode-array detection (HPLC/DAD). Recombinant human UGTs were purchased from BD Gentest (Woburn, USA). Pooled and individual HLMs were purchased from Rild Research Institute for Liver Diseases (Shanghai, China). All other solvents were purchased with HPLC grade.

Incubation System

The incubation system for the UGT reaction included HLM (5 mg/mL), UDPGA (40 mM), Tris-HCl buffer (PH 7.4), $MgCl_2$ (50 mM), 25 μ g/mL alamethicin, 10 mM D-saccharic acid 1,4-lactone, and substrates in a final volume of 200 μ L (23,24). Organic solvent was not more than 1% by volume. After 30-min incubation at 37°C, the reaction was terminated by the addition of 0.1 mL methanol, followed by centrifugation at 20,000 $\times$ g for 20 min. The supernatant was subjected to HPLC-UV-ESI analysis. Control incubations without UDPGA, substrate, or microsomes were used to ensure that metabolite formation was microsome- and UDPGA-dependent.

An Agilent 1200 HPLC system with quaternary delivery system, degasser, autosampler, UV-detector, and Elite SinoChorm ODS-BP (2.1 $\times$ 150 mm, 5 μ m) analytical column was used for quantification. The mobile phase consisted of acetonitrile–0.1% formic acid aqueous solution at a flow rate of 0.5 mL/min. An Applied Biosystems MDS Sciex API 3200 Triple Quadrupole Mass Spectrometer (MS/MS) equipped with electrospray ionization (ESI) source was used to analyze target metabolites. The system was operated in negative

mode with the following values: andrographolide (525.0→349.0), dehydroandrographolide (507.5→331.5) and deoxyandrographolide (509.0→333.0). The optimized ionspray voltage and temperature were set at 5,000 V and 600°C, respectively. The curtain gas (CUR) flow was 10 L/min; gas1 and gas2 (nitrogen) were set at 45 and 40 psi, respectively, and dwell time was 150 ms. Nitrogen was used as both curtain and collision gas, controlled at 13 and 6 psi, respectively. Quantification assay was performed using multiple reaction monitoring.

Biosynthesis of Metabolites and NMR Spectroscopy

The metabolites of AND, DEO, and DEH were biosynthesized *in vitro* using liver microsomes from different animal species. Glucuronides were isolated and purified for structure elucidation and quantitative analysis. Enzymatic reactions were carried out with PLM because they were found to efficiently catalyze the formation of all metabolites detected in other microsomal samples. In brief, 50 mM AND was incubated with PLM (5 mg/mL), 50 mM Tris-HCl (pH=7.4), 50 mM MgCl₂, and 40 mM UDPGA in 1 mL total volume for 6 h. DEO and DEH were incubated with CyLM under the same conditions as AND. Metabolites were isolated and purified by HPLC instrument with C₁₈ ODS column. The structures of all metabolites were determined by spectroscopic methods including 2D NMR (HSQC and HMBC). All spectra were recorded on a Bruker AV-600 (Bruker, Newark, Germany). Purified metabolites were stored at -20°C before NMR analysis in deuterated methanol (Euriso-Top, Saint-Aubin, France). Chemical shifts of ¹H and

¹³C signals and the C-H correlation between glucuronic acid and the parent nucleus were used to confirm the conjugation site. All the ¹H- and ¹³C NMR spectral data of these metabolites of AND, DEH, and DEO were unambiguously assigned by 2D NMR including HSQC and HMBC (Table I, Figs. S1 and S9).

Assay with Recombinant UGTs

AND (3, 30, and 300 μM), DEO (2, 20, and 100 μM), and DEH (1, 20, and 100 μM) were incubated with commercial recombinant UGT1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, and 2B17 in the standard incubation system. Three substrate concentrations were used in this study: the high and intermediate concentrations were the approximate *V*_{max} and *K*_m values for HLM, respectively. Low concentrations were used to evaluate the catalytic activity of the UGT isoform(s) with high affinity for glucuronidation. All assays were conducted at 37°C for 60 min at a final protein concentration of 0.5 mg/mL. HPLC-UV was used to monitor metabolites.

Correlation Study

AZT, a selective probe for UGT2B7, was used in the correlation study (25,26). HLMs from 11 individuals were used. The rates of glucuronidation of AND, DEH, and DEO were compared with AZT in these 11 HLMs *via* linear regression. The concentration of AZT was 500 μM (close to its *K*_m); HLM protein concentration was 0.5 mg/mL, and the reaction time was 30 min. AND, DEO, and DEH were

Table I. ¹³C NMR (150 MHz, MeOD) Spectral Data for Monoglucuronide Metabolites of AND, DEH, and DEO

No.	AND-1	AND-2	DEH-1	DEH-2	DEO-1	DEO-2
1	38.6	38.0	39.8	39.3	38.6	37.9
2	29.0	25.4	28.8	24.4	29.0	24.6
3	80.3	86.9	80.5	87.3	80.5	87.1
4	44.0	44.0	44.1	44.1	44.1	44.0
5	56.6	56.8	56.1	56.3	56.9	57.0
6	25.7	24.5	24.9	24.7	25.8	25.6
7	39.1	39.0	37.9	37.8	39.5	39.4
8	148.9	148.9	150.3	150.2	148.9	148.8
9	57.5	57.2	63.0	62.6	57.4	57.1
10	40.1	39.9	40.0	39.6	40.3	40.1
11	25.7	25.8	138.9	136.5	23.1	23.4
12	149.5	149.4	122.4	122.5	25.4	25.4
13	129.8	129.8	129.6	129.6	134.7	134.7
14	66.6	66.6	146.7	146.8	147.7	147.7
15	76.2	76.2	71.6	71.6	72.1	72.1
16	172.7	172.7	174.9	174.9	177.0	177.0
17	109.1	109.2	108.9	109.1	107.5	107.6
18	23.9	23.4	24.0	23.4	23.9	23.2
19	72.3	64.1	72.4	64.2	72.3	64.1
20	15.2	15.2	16.0	15.9	15.4	15.3
1'	105.1	101.8	105.2	101.9	105.1	101.9
2'	74.7	74.7	73.1	73.2	74.9	74.7
3'	77.6	77.4	77.6	76.8	77.5	77.3
4'	73.2	73.2	74.7	74.7	73.1	73.0
5'	76.3	76.8	76.6	77.4	76.6	76.5
6'	176.7	176.7	172.5	174.0	172.7	172.5

AND andrographolide, DEH dehydroandrographolide, DEO deoxyandrographolide

incubated with HLM (0.2 mg/mL protein concentration) for 15–25 min. Correlations were determined between expression level of UGT2B7 and AND, DEH, and DEO glucuronidation rate in 11 HLMs. UGT2B7 protein was quantitated by Western blot as described previously (27). Briefly, 10 µg HLM was spotted on 10% SDS–polyacrylamide gel and transferred electrophoretically to either polyvinylidene difluoride or nitrocellulose membranes. Polyvinylidene difluoride membrane Immobilon-P (Millipore Corporation, Billerica, MA) was probed with antihuman UGT2B7 antibody. Quantitative analysis was performed using a GT-9800F scanner (Seiko Epson, Suwa, Japan) and ImageQuant TL Image Analysis software (GE Healthcare). Correlation analysis for HLMs from 11 donors was determined by Spearman's rank method. When the *r* value was greater than or equal to 0.5 and the *P* value was less than 0.05, the correlations were considered statistically significant.

Chemical Inhibition Study

AND, DEO, and DEH were incubated with HLM in the presence or absence of UGT-specific inhibitors: UGT2B7 inhibitors fluconazole (5 mM), diclofenac (400 µM), and ppt (100 µM) as well as UGT1A inhibitor phenylbutazone (500 µM) (24,28,29). To confirm the role of UGT2B7 in glucuronidation of AND, DEO, and DEH, HLMs and UGT2B7 were treated with diclofenac (0–300 µM). IC₅₀ values were determined as described previously (30).

Kinetic Study

For estimating kinetic parameters, AND (1–500 µM), DEO (1–300 µM), and DEH (1–500 µM) were incubated with pooled microsomes or recombinant human UGT1A4, 2B4, or 2B7. To ensure that less than 10% of substrate was metabolized, incubation times and protein concentrations were selected within the linear interval of metabolite turnover rates. Kinetic models were used to analyze the results: Michaelis–Menten (Eq. 1) (31), biphasic (Eq. 2) (31), and inhibition kinetics (Eq. 3) (30).

$$v = V_{\max} \times S / (K_m + S) \quad (1)$$

$$v = V_{\max 1} \times S / (K_{m1} + S) + V_{\max 2} \times S / (K_{m2} + S) \quad (2)$$

$$v = \frac{V_{\max}[S]}{K_m + [S] + [S]^2/K_i} \quad (3)$$

$V_{\max}$ represents the maximum rate and K_m is the substrate concentration at half-maximal rate. All incubations were performed in three independent experiments in duplicate. Kinetic constants were obtained using Origin 7.5 (OriginLab Corp., Northampton, MA) and are reported as the mean ± SE.

Assay for Inhibition of Cellular NO Production

RAW 264.7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal

bovine serum and 1% antibiotics under 5% CO₂ at 37°C. Cells were activated with LPS. Briefly, cells were plated in 96-well plates (2 × 10⁵ cells/well). After preincubation for 2 h with test compounds (0–100 µM), LPS (1 µg/mL) was added and incubated for 18 h unless otherwise specified. Test compounds dissolved in DMSO were diluted to the appropriate concentration with serum-free DMEM. The final concentration of DMSO was adjusted to 0.1%. To assess NO production, the stable conversion product of NO was measured using the Griess reagent and the optical density was determined at 540 nm (Table S4).

RESULTS

Identification of Metabolites in Microsomal Samples

Two monoglucuronides of andrographolide (AND-1 and AND-2) were detected in CyLM and PLM samples, while only AND-1 was detected in HLM, DLM, and MLM. ESIMS of AND-1 provided an [M–H][–] ion peak at *m/z* 525.1, suggesting a molecular formula C₂₆H₃₈O₁₁. The carbon signals at δ105.1, 74.7, 77.6, 73.2, 76.3, and 176.7 indicated that a β-D-glucuronic acid was introduced (Table I). The ¹H- and ¹³C NMR spectral data agreed well with those reported in the literature (11). Thus, AND-1 was identified as andrographolide-19-*O*-β-D-glucuronide, previously isolated from human urine. In addition, ESIMS of AND-2 also gave an [M–H][–] peak at *m/z* 525.0, suggesting the molecular formula C₂₆H₃₈O₁₁. Compared with AND, the ¹³C NMR spectrum of AND-2 included six additional carbon signals and a large upfield shift (Δδ +6.6) at C-3. Further, the HMBC spectrum showed strong correlations of H-3 (δ3.54) with C-1' (δ101.8), C-2 (δ25.4), and C-19 (δ64.1). This is strong evidence that a β-D-glucuronide group had been introduced at C-3 (Table I). Therefore, AND-2 was identified as andrographolide-3-*O*-β-D-glucuronide, a new metabolite of AND.

Two novel metabolites (DEH-1 and DEH-2) were found in CyLM, PLM, RLM, and MLM samples, while only DEH-1 was detected in liver microsomal samples from human and dog. ESIMS of DEH-1 and DEH-2 gave a *quasi*-molecular ion [M–H][–] at *m/z* 507.1, corresponding to the molecular formula C₂₆H₃₆O₁₀. The ¹³C NMR spectrum of DEH-1 showed six additional carbon signals (δ105.2, 73.1, 77.6, 74.7, 76.6, and 172.5) and a large upfield shift (Δδ +8.2) at C-19, indicating that DEH-1 was dehydroandrographolide-19-*O*-β-D-glucuronide. The ¹³C NMR spectrum of DEH-2 showed six additional carbon signals and an upfield shift (Δδ +6.8) at C-3 (Table I and Table S1), suggesting that DEH-2 is dehydroandrographolide-3-*O*-β-D-glucuronide.

For DEO, two new monoglucuronides (DEO-1 and DEO-2) were detected in CyLM and PLM samples, while only DEO-1 was observed in HLM, DLM, RLM, and MLM, as determined by ESIMS ([M–H][–] *m/z* 509). Compared with DEO, six additional carbon signals (δ105.1, 74.9, 77.5, 73.1, 76.6, and 172.7) were observed in the ¹³C NMR spectra of DEO-1 and DEO-2. Further, large upfield shifts were observed at C-19 in DEO-1 (Δδ +7.9) and C-3 in DEO-2 (Δδ +7.2). These results indicate that a β-D-glucuronide moiety was conjugated to C-19 of DEO-1 and C-3 of DEO-2. Thus, DEO-1 and DEO-2 were identified as

deoxyandrographolide-19-*O*- β -D-glucuronide and deoxyandrographolide-3-*O*- β -D-glucuronide, respectively.

Glucuronidation Assay with Recombinant Human UGTs

Reaction phenotyping was performed using twelve recombinant human UGT isoforms (UGT 1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B15, and 2B17) to identify the main UGT isoforms involved in glucuronidation of AND, DEH, and DEO. At three different substrate concentrations, UGT1A3, 1A4, 2B4, and 2B7 showed glucuronidation activity toward AND, DEH, and DEO. The glucuronidation rates of AND (30 μ M, 10 K_m in HLMs) were 94, 1.2, 19, and 4.7 pmol min⁻¹ mg⁻¹ for UGT2B7, 2B4, 1A4, and 1A3, respectively. Similarly, the glucuronidation rates of DEH and DEO were 0.57 and 0.26 nmol/min/mg for UGT 2B7, much higher than for UGT1A4, 1A3, or 2B4 (Fig. 2). Overall, UGT2B7 showed the highest catalytic activity toward AND, DEH, and DEO. However, at the highest

concentration (300 μ M, 100 K_m), UGT1A4 exhibited higher catalytic activity toward AND than UGT2B7.

Chemical Inhibition Study

The effects of fluconazole, phenylbutazone, ppt, and diclofenac on glucuronidation of AND and its derivatives (DEO and DEH) were determined using HLM. Considering the fact of substrate-dependent inhibition for UGTs, several UGT2B7 inhibitors were used. At three different substrate concentrations, ppt and diclofenac strongly inhibited glucuronidation of AND and its derivatives with a residual activity of 12–56% (Fig. S2). In contrast, inhibition of the glucuronidation of AND, DEH, and DEO by fluconazole and phenylbutazone was negligible in each case. Although strong inhibition of UGT2B7 by fluconazole was reported previously, the discrepancy might be due to different substrate (4-methylumbelliferone vs. AND) used in the two individual studies. As reported, diclofenac was used as a selective inhibitor of UGT2B7 (30). We examined the effects of diclofenac

Fig. 2. Glucuronidation of andrographolide (a), deoxyandrographolide (b), and dehydroandrographolide (c) by various recombinant UGTs at the different concentrations

on glucuronidation by UGT2B7. Diclofenac had similar potency in inhibition of HLM- and UGT2B7-mediated glucuronidation, at substrate concentrations close to K_{m1} . IC_{50} values are shown in Table S3.

Correlation Study

Glucuronidation rates for AZT (a selective probe for UGT2B7), AND, DEO, and DEH were determined using individual human liver microsomes ($n=11$). Further, the expression levels of UGT2B7 in individual HLMs were measured by Western blot. Finally, the correlation analyses of AND, DEO, and DEH glucuronidation activities with AZT glucuronidation activity or with expression of UGT2B7 isoform in these 11 individuals as determined by Western blot were performed. As shown in Figs. 3 and S3, the glucuronidation rates of AND, DEO, and DEH showed significant correlations with UGT2B7 expression, with correlation coefficients (R) in the range of 0.90 to 0.94 ($P<0.05$), especially at low substrate concentrations (around K_m). These results demonstrate that UGT2B7 plays a dominant role in 19-*O*-glucuronidation of AND and its analogs in HLMs.

Kinetic Characterization of AND, DEO, and DEH Glucuronidation in HLM and Recombinant UGT Isoforms

Kinetic analyses were performed for HLMs and recombinant UGT2B7, 1A4, and 2B4 (Figs. 4 and S4). The kinetic parameters for AND-1, DEH-1, and DEO-1 in HLM and UGT isoforms are listed in Table II. Total intrinsic clearances (CL_{int}) *in vitro* (AND-1, DEH-1, and DEO-1, calculated as V_{max}/K_m) for 19-*O*-glucuronidation in HLM and UGT isoforms are shown in Table III. The Michaelis–Menten model gave the best fit to 19-*O*-glucuronidation of DEH in HLM. K_m and V_{max} were $22.1 \pm 1.48 \mu M$ and $2.06 \pm 0.03 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ protein}$, respectively, very similar to the intrinsic clearances (CL_{int}) of UGT2B7 (93.2 vs. $97.8 \mu L \text{ min}^{-1} \text{ mg}^{-1}$). In contrast, AND glucuronidation by HLM followed biphasic kinetics ($K_{m1}=3.06 \pm 0.31 \mu M$, $V_{max1}=0.21 \pm 0.006 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ protein}$; $K_{m2}=1,110 \pm 510 \mu M$, $V_{max2}=0.540 \pm 0.171 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ protein}$). UGT2B7 had much higher affinity for AND than UGT1A4; intrinsic clearances (CL_{int}) for UGT2B7 and HLM were close (69.2 vs. $93.6 \mu L \text{ min}^{-1} \text{ mg}^{-1}$). Collectively, these results imply that UGT2B7 is chiefly responsible for 19-*O*-glucuronidation of AND in HLM. HLM exhibited strong affinity toward DEO (K_m $108 \pm 22.6 \mu M$ and $2.11 \pm 0.520 \mu M$), exhibiting biphasic kinetics in 19-*O*-glucuronidation. Compared to UGT2B4 and 1A4, UGT2B7 showed higher affinity ($K_m=1.50 \pm 0.07 \mu M$) and larger CL_{int} ($169 \mu L \text{ min}^{-1} \text{ mg}^{-1}$). CL_{int} (AND-1, DEH-1, and DEO-1, calculated as V_{max}/K_m) for 19-*O*-glucuronidation by HLM and UGT isoforms are shown in Tables II and III. We were unable to estimate the kinetic parameters for UGT1A3-mediated glucuronidation due to the low rate.

Interaction of Andrographolide and Its Two Derivatives

Enzyme-inhibition studies indicated that inhibition of AND glucuronidation by either DEO or DEH was

concentration-dependent with IC_{50} values of 6.7 and 45.1 μM , respectively. Dixon and Lineweaver–Burk plots were used to identify the inhibition kinetic type (Fig. S5). The slopes obtained from Lineweaver–Burk plots against concentrations of DEO or DEH were used to calculate K_i values. Based on the corresponding interaction point, DEO and DEH competitively inhibited glucuronidation of AND, with K_i values of 2.5 and 12.8 μM , respectively.

Inhibitory Effects on NO Production of Three Drugs and Their Metabolites

AND, DEO, and DEH and their metabolites were quantified based on cytotoxicity against RAW 264.7 macrophages. Only AND was slightly toxic, with cell viability of 57.5% at 100 μM (Table S4). LPS (1.0 $\mu g/mL$) increased NO production compared with the control group ($P<0.001$). Minocycline (MINO) was used as the positive control for NO production, with IC_{50} value of 39.9 μM . All compounds were tested for inhibition of LPS-induced NO production in RAW 264.7 macrophages at 1–100 μM , using the Griess method to evaluate anti-inflammatory activity. Cell viability was not affected at 100 μM , which implies that glucuronidation may reduce toxicity. AND gave the strongest inhibition of LPS-induced NO production, followed by AND-1, DEO, DEH, and the 19-*O*-glucuronides of DEH and DEO (Table S4). In contrast, the 3-*O*-glucuronides of DEH and DEO did not inhibit LPS-induced NO production (IC_{50} values over 100 μM). For the first time, this result strongly suggests that 19-*O*-glucuronidation is a critical step in the conversion of AND to its bioactive anti-inflammatory metabolite.

Glucuronidation of AND, DEH, and DEO in Animal Liver Microsomes

The UGT metabolic profiles indicated that 19-*O*-glucuronidation of AND, DEH, and DEO occurs in all species except rats, while 3-*O*-glucuronidation is substrate-dependent. 3-*O*-Glucuronidation occurred in CyLM and PLM for AND and DEO, and in CyLM, PLM and RLM for DEH. DLM and HLM had similar activities in 19-*O*-glucuronidation of AND, DEH, and DEO. Kinetic studies comparing glucuronidation of AND, DEH, and DEO (Table IV, Figs. S6 and S7) indicated that the formation of AND and DEH glucuronides followed Michaelis–Menten kinetics in all microsomal samples, except for DEH 19-*O*-glucuronidation in RLM and DEH 3-*O*-glucuronidation in PLM, where biphasic kinetics were observed. For 19-*O*- and 3-*O*-glucuronidation of DEO, substrate inhibition kinetics were observed in all microsomes except CyLM, which obeyed Michaelis–Menten kinetics. As shown in Tables III and S5, CL_{int} for 19-*O*-glucuronidation of AND was comparable in all species, while 3-*O*-glucuronidation was approximately 3.1 times higher in PLM than CyLM. DEH CL_{int} values were in the order, rat>monkey>mouse>dog>pig (19-*O*-glucuronidation) and pig>rat>monkey>mouse (3-*O*-glucuronidation). In contrast, the CL_{int} values for DEO were in the order, mouse>monkey>pig>dog>rat (19-*O*-glucuronidation) and monkey>pig (3-*O*-glucuronidation).

Fig. 3. Western blots of recombinant UGT2B7 and human liver microsomes (a). Panels b–d: the correlation analyses ($n=11$) were performed between the UGT2B7 protein and AND glucuronidation (a), DEH glucuronidation (b), and DEO glucuronidation (c). Correlation coefficients were calculated as well as $P < 0.05$.

Fig. 4. The kinetic profiles of 19-*O*-glucuronidation of andrographolide, deoxyandrographolide, and dehydroandrographolide for HLM (a), human recombinant UGT2B7 (b), UGT2B4 (c), and UGT1A4 (d), respectively. Each data point represents the average of three replicates. Experiment details were shown in the experimental section

DISCUSSION

In a previous investigation (10), 13 metabolites of AND generated by pooled HLMs, DLMs, and RLMs were detected by LC-MS/MS. *In vitro* metabolic reactions of AND including glucuronidation, dehydration, and deoxygenation were inferred from MS/MS fragmentation. Nevertheless, the exact

location of conjugation could not be established, especially for glucuronidation. In the present study, we identified for the first time the structures of glucuronides of AND, DEO, and DEH and determined their kinetics of formation. The hydroxyl group at C-19 was found to be the major site for glucuronidation of AND, DEO, and DEH in HLMs. These findings are consistent with a previous report, which showed

Table II. Kinetic Parameters for Glucuronidation of AND, DEH, and DEO in HLMs, and Recombinant UGT2B7, UGT2B4, and UGT1A4. $V_{\max}$ nmol/min/mg protein, K_m μM . ($n=3$)

Enzyme	AND				DEH		DEO			
	$V_{\max 1}$	$V_{\max 2}$	K_{m1}	K_{m2}	$V_{\max}$	K_m	$V_{\max 1}$	$V_{\max 2}$	K_{m1}	K_{m2}
HLM	0.210 ± 0.011	0.540 ± 0.171	3.06 ± 0.31	$1,110 \pm 510$	2.06 ± 0.033	22.1 ± 1.48	0.680 ± 0.040	0.710 ± 0.050	108 ± 22.6	2.11 ± 0.520
UGT2B7	0.142 ± 0.010	—	1.46 ± 0.08	—	1.06 ± 0.013	10.8 ± 0.420	0.253 ± 0.014	—	1.50 ± 0.07	—
UGT2B4	—	—	—	—	0.233 ± 0.012	69.9 ± 3.09	0.071 ± 0.013	—	65.1 ± 5.74	—
UGT1A4	0.732 ± 0.191^a	—	$1,020 \pm 357^a$	—	—	—	0.191 ± 0.013	—	123 ± 14.4	—

AND andrographolide, DEH dehydroandrographolide, DEO deoxyandrographolide, HLM human liver microsomes, UGT UDP-glucuronosyltransferase

^a The approximate $V_{\max}$ and K_m values of UGT1A4 for AND (the maximal solubility of AND in incubation system was 500 μM)

Table III. Intrinsic Clearances (CL_{int}) of 19-*O*- β -Glucuronidation of AND, DEH, and DEO in HLM, Isoforms, and Different Species. CL: $\mu\text{L}/\text{min}/\text{mg}$. ($n=3$)

	CL_{AND}	CL_{DEH}	CL_{DEO}		CL_{AND}	CL_{DEH}	CL_{DEO}
HLM (K_{m1})	69.2	93.2	6.32	Pig	26.6	47.7	195
HLM (K_{m2})	0.490	–	335	Dog	25.7	215	143
UGT2B7	93.6	97.8	169	Monkey	25.2	556	249
UGT 2B4	–	3.29	1.02	Rat (K_{m1}/K_{m2})	–	73.7/991	122/–
UGT 1A4	0.711	–	1.54	Mouse	22.7	233	1,010

AND andrographolide, DEH dehydroandrographolide, DEO deoxyandrographolide, HLM human liver microsomes, UGT UDP-glucuronosyltransferase

that the 19-*O*-glucuronide conjugate of AND was the major metabolite in human urine (11). In addition, to the best of our knowledge, we were the first to report 19-*O*-glucuronides in human metabolism of DEO and DEH, which are found in high levels in chuanxinlian table and its combinations (22,32). Many AND derivatives were synthesized in previous studies (33–36). To improve the poor bioavailability of AND and derivatives caused by first-pass glucuronidation, an effective approach is to remove or block the vulnerable metabolic sites. Our findings suggest that the replacement of the 19-OH group by methoxy or acetyl groups would enhance metabolic stability, preventing premature glucuronidation. Knowledge of the glucuronidation sites of AND, DEH, and DEO will be useful for structural modification of AND and its derivatives in future drug development, permitting enhancement of metabolic stability.

Animal models have been commonly used in preclinical studies to predict pharmacokinetics and toxicity in humans. A suitable animal models used for preclinical studies should have metabolic patterns similar to humans, including identical or similar metabolic activities, enzymes, and catalytic processes (37). An *in vitro* study by Zhao *et al.* (10) attempted to evaluate the broad differences in CYP-mediated metabolism of HLMs, DLMs, and RLMs rather than differences between UGTs. However, to the best of our knowledge, glucuronidation is considered the dominant *in vivo* human metabolic pathway for AND (11). Here, a comparative study of AND, DEH, and DEO glucuronidation in humans and five common experimental animal species was performed to determine the catalytic efficiency of glucuronidation in liver microsomes. Our results suggest that the 19-*O*-glucuronides of AND, DEO, and DEH appear to be common to all species, as observed for CyLM, PLM, RLM, and MLM.

Intrinsic clearance (CL_{int}) of the 19-*O*-glucuronide as a unique UGT metabolic pathway in HLM should be taken into account in selection of animal models. These findings indicate that pigs may be a preferred surrogate model for metabolic and pharmacokinetic studies of AND because CL_{int} values were similar to human values (69.6 vs. 26.6 $\mu\text{L}/\text{min}/\text{mg}$). Similarly, we could consider the monkey model for DEO (335 vs. 249 $\mu\text{L}/\text{min}/\text{mg}$) and pig model for DEH (93.2 vs. 47.7 $\mu\text{L}/\text{min}/\text{mg}$), in extrapolating pharmacokinetic and toxicological results to humans (Table III). However, most pharmacological studies on AND and its derivatives in the past used rat. Due to a lack of comparable data on 19-*O*-glucuronidation of AND and its derivatives in HLM, previous results obtained in rats should be treated with caution, especially in the case of bioactive metabolites.

Multiple UGT isoforms (including UGT1A3, UGT1A4, UGT2B4, and UGT2B7) exhibited glucuronidation activity toward AND, DEH, and DEO. Based on the expression of all these UGT isoforms in human liver, kinetic studies were employed to identify the major UGT isoform responsible for their glucuronidation. Among these UGT isoforms, UGT2B7 showed the highest affinity for AND, DEH, and DEO, with K_m values of 1.46, 10.8, and 1.50 μM , respectively. The CL_{int} values for UGT2B7 (93.6 to 169 $\mu\text{L}/\text{min}/\text{mg}$) were also much higher than for UGT1A4 and 2B4 (0.710 to 3.29 $\mu\text{L}/\text{min}/\text{mg}$), strongly suggesting that UGT2B7 is mainly responsible for glucuronidation of AND, DEH, and DEO in HLMs. Further, high correlations of glucuronidation rate with expression of UGT2B7 in HLMs, and systemic inhibition studies in HLMs and UGT2B7, strongly suggest that UGT2B7 plays a predominant role in 19-*O*-glucuronidation of AND, DEH, and DEO. To our

Table IV. Kinetic Parameters of 19-*O*- β -Glucuronidation Derived from Incubation of AND, DEH, and DEO with Various Species' Liver Microsomes. ($n=3$)

Species	AND		DEH				DEO		
	V_{max}	K_m	V_{max1}	V_{max2}	K_{m1}	K_{m2}	V_{max}	K_m	K_i
	$\text{nmol}/\text{min}/\text{mg protein}$	μM	$\text{nmol}/\text{min}/\text{mg protein}$		μM		$\text{nmol}/\text{min}/\text{mg protein}$	μM	μM
Pig	1.35 $\pm$ 0.02	50.9 $\pm$ 2.38	1.67 $\pm$ 0.03	–	35.1 $\pm$ 1.95	–	3.88 $\pm$ 0.10	19.9 $\pm$ 1.34	993 $\pm$ 108
Dog	0.770 $\pm$ 0.011	30.0 $\pm$ 1.91	3.09 $\pm$ 0.02	–	14.4 $\pm$ 0.380	–	1.95 $\pm$ 0.041	13.7 $\pm$ 0.86	1,370 $\pm$ 164
Monkey	2.34 $\pm$ 0.062	931 $\pm$ 6.80	15.5 $\pm$ 0.330	–	27.9 $\pm$ 2.30	–	4.34 $\pm$ 0.050	17.4 $\pm$ 1.12	–
SD rat	–	–	2.45 $\pm$ 0.50	1.92 $\pm$ 0.581	33.2 $\pm$ 16.5	1.93 $\pm$ 0.950	2.27 $\pm$ 0.070	18.7 $\pm$ 1.60	1,280 $\pm$ 208
Mouse	1.56 $\pm$ 0.031	68.7 $\pm$ 4.25	1.76 $\pm$ 0.042	–	7.55 $\pm$ 0.881	–	16.1 $\pm$ 0.460	15.9 $\pm$ 1.35	2,490 $\pm$ 628

AND andrographolide, DEH dehydroandrographolide, DEO deoxyandrographolide

knowledge, UGT2B7 is an important UGT isoform with a high expression level in human liver. Its catalytic activity varies widely depending on the individual, environmental, genetic, and other factors (19,25,26,38,39). Therefore, metabolism and pharmacokinetics of AND, DEH, and DEO would vary among individuals with a different UGT2B7 genotype. Our results suggest that caution should be exercised in their clinical use, in combination with other drugs primarily metabolized by UGT2B7 (e.g., NSAIDs, morphine, and codeine) (25,39). This may create a risk of drug–drug interactions.

The affinity and catalytic activity of metabolic enzymes are closely related to the chemical structures of their substrates. This study shows that AND, DEO, and DEH, structurally related ent-labdane diterpenoids, are metabolized mainly by UGT2B7. Kinetic studies showed that AND and DEO had 7-fold higher affinity toward UGT2B7 than did DEH. Additionally, the K_m value of 3-oxo-DEH in UGT2B7 was $22.1 \pm 1.41 \mu\text{M}$ (Fig. S6), indicating that a double bond at C-11 and C-12 in DEH reduces affinity for UGT2B7. In the case of UGT2B4, a hydroxy substituent in the furan rings of AND may hinder binding to the active site of UGT2B4. Since a 3D structure is unavailable for human UGT enzymes (16), our findings presented herein will be an important supplement to understand the role of the active sites in UGT2B7 as well as UGT2B4.

Most UGTs have broad and overlapping substrate specificity; few highly substrate-selective UGT isoform(s) have been reported. Morphine, codeine, and AZT are recommended as UGT2B7 probe substrates in previous reports (25,26). Compared with these substrates, AND, DEO, and DEH have higher affinity toward this UGT isoform. Furthermore, the CL_{int} values of three drugs for UGT2B7 were more than 100-fold higher than those for UGT1A4 and 2B4, suggesting better selectivity for UGT2B7. Additionally, DEH 19-*O*-glucuronidation rates correlated strongly with expression of UGT2B7 in HLMs from 11 individuals, as determined by Western blot analysis. However, the selectivity of morphine, codeine, and AZT for UGT2B7 is relatively low (25). Besides high selectivity and catalyzing activity for enzymes, a probe substrate with excellent Michaelis–Menten kinetics should also be required (40). On these criteria, DEH may be a more promising *in vitro* substrate of UGT2B7 than AND and DEO.

In most cases, phase II metabolism inactivates or detoxifies drugs (15,16). Our bioassay indicated that 19-*O*-glucuronides of AND and its derivatives showed moderate anti-inflammatory activity. These findings indicate that in addition to the parent drug, the 19-*O*-glucuronide should be considered in evaluating their efficacy against inflammatory diseases. To some extent, the role 19-*O*-glucuronidation of AND, DEO, and DEH in the *in vivo* anti-inflammatory effects of these compounds has been underestimated previously.

AND, an anti-inflammatory agent, has been widely used in clinical practice for the treatment of inflammation and infectious diseases. Fixed-dose combinations of AND with DEH and DEO are widely prescribed in China, India, and other Asian countries. Therefore, interactions of them are most likely to occur *via* inhibition of UGT2B7 that primarily catalyzes 19-*O*-glucuronidation. As expected, DEO and DEH

were found to be competitive inhibitors of AND glucuronidation with K_i values of 2.5 and 12.8 μM , respectively. For reversible inhibition, an *in vivo* interaction *via* the inhibition of UGT2B7 would possibly occur if C_{max}/K_i greater than 0.1 (C_{max} is the inhibitor level at steady state and at the highest clinical dose). The human C_{max} of DEH after a single oral dose of Chuanxinlian tablet (0.42 g) was approximately 147.3 $\mu\text{g/L}$ (equal to 0.42 μM). Thus, the C_{max}/K_i value for DEH was less than the FDA's cutoff of 0.1, suggesting little risk of inhibition *in vivo*. The C_{max} value of DEO in humans is unavailable. However, the risk of inhibition is increased by its relatively low K_i value and its higher content than DEH in Chuanxinlian (22).

In conclusion, in this study, we report the first analysis of the glucuronidation of AND, DEH, and DEO in liver microsomes prepared from humans and five experimental animal species. UGT2B7 was responsible for formation of 19-*O*-glucuronides from AND, DEH, and DEO. It was particularly notable that the 19-*O*-glucuronide of AND in HLM exhibited potent anti-inflammatory activity. Additionally, DEH may be used as an *in vitro* substrate for UGT2B7 due to its high selectivity. Further, significant species differences in glucuronidation were related to similarities in metabolic profile and catalytic efficacy. These findings may assist in selection of the right animal models for pharmacokinetic or toxicological studies of AND, DEH, and DEO. Our findings may also provide useful guidance for clinical use and for the development of new anti-inflammatory agents from AND derivatives.

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