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Comparison of 1beta- and 5beta-hydroxylation of Deoxycholate and Glycodeoxycholate as
In Vitro Index Reactions for CYP3A Activities

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CYP3A-Catalyzed Deoxycholate and Glycodeoxycholate Oxidation

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Abbreviations

BA, bile acid
CA, cholic acid, 3α, 7α, 12α-trihydroxy-5β-cholan-24-oic acid
CDCA, chenodeoxycholic acid, 3α, 7α-trihydroxy-5β-cholan-24-oic acid
DCA, deoxycholic acid, 3α, 12α-dihydroxy-5β-cholan-24-oic acid
1βh-DCA, 1β-hydroxydeoxycholic acid, 1β, 3α, 12α-trihydroxy-5β-cholan-24-oic acid
5βh-DCA, 5β-hydroxydeoxycholic acid, 3α, 5β, 12α-trihydroxy-5β-cholan-24-oic acid
GDCA, glycodeoxycholic acid, N-(3α, 12α-dihydroxy-cholan-24-oyl) glycine
1βh-GDCA, 1β-hydroxyglycodeoxycholic acid, N-(1β, 3α, 12α-trihydroxy-cholan-24-oyl)-glycine
5βh-GDCA, 5β-hydroxyglycodeoxycholic acid, N-(3α, 5β, 12α-trihydroxy-cholan-24-oyl)-glycine
TDCA, taurodeoxycholic acid, N-(3α, 12α-dihydroxy-cholan-24-oyl)-taurine
GUDCA-D4, glycoursodeoxycholic acid-D4, N-(3α, 7β-dihydroxy-cholan-24-oyl-2,2,4,4-d4)-glycine
DMF, N, N-dimethylformamide
DMT-MM, 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride
MDZ, midazolam
1′h-MDZ, 1′-hydroxymidazolam
4h-MDZ, 4-hydroxymidazolam
CLZ, clozapine
T, testosterone
2βh-T, 2β-hydroxytestosterone
6βh-T, 6β-hydroxytestosterone
15βh-T, 15β-hydroxytestosterone
AD, androstenedione
4h-AD, 4-hydroxyandrostenedione
PROG, progesterone
HLM, human liver microsomes
rP450s, recombinant CYP450 enzymes
CYP3A, Cytochrome P450 3A
PBS, phosphate buffer saline
NADP(H), nicotinamide adenine dinucleotide phosphate
LC-MS/MS, liquid chromatography with tandem mass spectrometry
MM, Michaelis-Menten
Hill, Allosteric sigmoidal
$S_{50}$, substrate concentration occupying half of the binding sites
$V_{\text{max}}$, maximal velocity
$\text{CL}_{\text{int}}$, intrinsic clearance
NA, not applicable
ND, not detected
BAAT, amino acid N-acyltransferase enzyme
DDI, drug-drug interaction
$^1\text{H-NMR}$, $^1\text{H}$ nuclear magnetic resonance
Abstract

Cytochrome P450 3A (CYP3A) participates in the metabolism of more than 30% of clinical drugs. The vast intra- and inter-individual variations in CYP3A activity pose great challenges to drug development and personalized medicine. It has been disclosed that human CYP3A4 and CYP3A7 are exclusively responsible for the tertiary oxidations of deoxycholic acid (DCA) and glycodeoxycholic acid (GDCA) regioselectivity at C-1β and C-5β. This work aimed to compare the 1β- and 5β-hydroxylation of DCA and GDCA as potential in vitro CYP3A index reactions in both human liver microsomes and recombinant P450 enzymes. The results demonstrated that the metabolic turnover of DCA 1β- and 5β-hydroxylation was 5-10 times higher than that of GDCA, suggesting that 1β-hydroxyglycodeoxycholic acid and 5β-hydroxyglycodeoxycholic acid may originate from DCA oxidation followed by conjugation in human. Metabolic phenotyping data revealed that DCA 1β-hydroxylation, DCA 5β-hydroxylation, and GDCA 5β-hydroxylation were predominantly catalyzed by CYP3A4 (>80%), while GDCA 1β-hydroxylation had approximately equal contributions from CYP3A4 (41%) and 3A7 (58%). Robust Pearson correlation was established for the intrinsic clearance of DCA 1β- and 5β-hydroxylation with midazolam (MDZ) 1’- and 4-hydroxylation in fourteen single donor microsomes. Although DCA 5β-hydroxylation exhibited a stronger correlation with MDZ oxidation, DCA 1β-hydroxylation exhibited higher reactivity than DCA 5β-hydroxylation. It is therefore suggested that DCA 1β- and 5β-hydroxylations may serve as alternatives to T 6β-hydroxylation as in vitro CYP3A index reactions.

Keywords

Deoxycholic acid; Glycodeoxycholic acid; tertiary bile acids; cytochrome P450 3A enzymes; index reaction.
Significance Statement

The oxidation of DCA and GDCA is primarily catalyzed by CYP3A4 and CYP3A7. This work compared the 1β- and 5β-hydroxylation of DCA and GDCA as in vitro index reactions to assess CYP3A activities. It was disclosed that the metabolic turnover of DCA 1β- and 5β-hydroxylation was 5-10 times higher than that of GDCA. Although DCA 1β-hydroxylation exhibited higher metabolic turnover than DCA 5β-hydroxylation, DCA 5β-hydroxylation demonstrated stronger correlation with MDZ oxidation than DCA 1β-hydroxylation in individual liver microsomes.
Introduction

Cytochrome P450 3A (CYP3A) is one of the most important subfamilies of P450 enzymes involved in the metabolism of both endogenous steroids and about 30% of clinical drugs (Guengerich, 1999; Zanger and Schwab, 2013). The human CYP3A subfamily comprises four genes: CYP3A4, CYP3A5, CYP3A7, and CYP3A43. CYP3A43 most likely does not translate into active enzyme (Westlind et al., 2001). CYP3A7 is expressed in the livers of fetuses and newborns. CYP3A7 expression starts to decline immediately after birth and is accompanied by an increase in CYP3A4 expression, the primary P450 isoform in adult liver. Recent studies have shown that CYP3A7 should not be neglected in adults since it is expressed in approximately 10% of adult livers (Burk et al., 2002; Sim et al., 2005; Couto et al., 2019). CYP3A5 exhibits similar transcription levels in the liver during all stages of development. The expression of CYP3A is highly variable through epigenetic mechanisms (Jin and Zhong, 2023) and activation of nuclear receptors (Guengerich, 1999). Drugs, natural products and environmental pollutants can also inhibit CYP3A (Liu et al., 2007). As a result, there are significant intra- and inter-individual difference in CYP3A activity, posing a great challenge in maintaining desired drug exposure in populations (Ingelman-Sundberg et al., 2007). Drug development and personalized medicine require sensitive, accurate, and convenient methods to predict or assess CYP3A activity in vitro and in vivo.

Genotyping and phenotyping play crucial roles in personalized clinical medication, assessment of the risk of severe adverse drug reactions, facilitation of new drug development, and evaluation of novel medications. Genotyping has limitations in predicting CYP3A activity due to its inability to account for the influence of environmental factors. Phenotyping serves as the efficient and direct approach to assess CYP3A activity by using a probe drug or index reaction that is metabolized or catalyzed by CYP3A. Some endogenous substances specifically metabolized by CYP3A have been investigated as potential CYP3A biomarkers (Ged et al., 1989; Kasichayanula et al., 2014). Kenworthy identified the relationships between 10 commonly used CYP3A probes and recommended the use of multiple probe reactions for in vitro evaluation of CYP3A activity (Kenworthy et al., 1999). Surveys conducted by reviewers in Center for Drug Evaluation and Research indicated that nearly 50% of studies selected testosterone (T) as the probe substrate for in vitro evaluation of CYP3A activity, while 15-20% of studies reported the application of midazolam (MDZ) in predicting in vitro CYP3A activity (Yuan et al., 1999; Yuan...
et al., 2002). Since then, MDZ 1'-hydroxylation and T 6β-hydroxylation are widely accepted as two complementary index reactions to assess the in vitro activity of CYP3A4 and 3A5.

Deoxycholic acid (DCA) is a secondary bile acid (BA) produced by gut bacteria via 7-dehydroxylation of cholic acid (CA) (Funabashi et al., 2020). DCA undergoes extensive hepatic conjugation with glycine or taurine into glycodeoxycholic acid (GDCA) or taurodeoxychoic acid (TDCA). Glycine conjugation is prevailing in adults while taurine conjugates exist primarily in fetus and neonates. More than 98% of BAs excreted from the liver are amidated (Russell, 2003). The hepatic synthesis of bile acid-amino acid conjugates involves two enzymatic reactions catalyzed by bile acid-CoA synthetase and bile acid-CoA: amino acid N-acyltransferase (Solaas et al., 2000). CYP3A4 and CYP3A7 are exclusively responsible for the tertiary oxidations of DCA, GDCA, and TDCA regioselectivity at C-1β, C-6α, C-5β, et al (Zhang et al., 2019). The C-1β and C-5β oxidized products seem to exist in human mainly as 1β-hydroxyglycodeoxycholic acid (1βh-GDCA) and 5β-hydroxyglycodeoxycholic acid (5βh-GDCA) and tend to undergo renal excretion without significant glucuronidation and/or sulfation (Zhang et al., 2019). As shown in Figure 1, 1βh-GDCA and 5βh-GDCA can be either products of GDCA oxidation or products of DCA oxidation reaction followed by conjugation. Comparison of the in vitro oxidation activity of DCA and GDCA may help to understand the main metabolic pathway of 1βh-GDCA and 5βh-GDCA.

Previous data showed that DCA oxidation activities correlate well with T 6β-hydroxylation activities in individual liver microsomes at single substrate level (Chen et al., 2019). Subsequent studies of species differences revealed that beagle dogs have a similar BA metabolism as humans compared to murine animals (Lin et al., 2020). Subsequent in vivo evaluation showed that the serum 1β-hydroxylation and 5β-hydroxylation ratios of DCA correlated well with oral MDZ clearance in beagle dogs, which has provided a proof-of-concept evidence that the tertiary oxidation of DCA is predictive of the in vivo CYP3A activity (Zeng et al., 2021). In this work, we prepared 1β-hydroxydeoxycholic acid (1βh-DCA), 5β-hydroxydeoxycholic acid (5βh-DCA), 1βh-GDCA and 5βh-GDCA and compared the 1β- and 5β-hydroxylation activities of DCA and GDCA in human liver microsomes (HLM) and recombinant human P450 enzymes (rP450s). It was disclosed that DCA 1β- and 5β-hydroxylation activities were much higher than those of GDCA, indicating that the synthesis of 1βh-GDCA and 5βh-GDCA might come from DCA oxidation followed by conjugation. Subsequent correlation analysis of oxidation activities of
DCA, GDCA, T and MDZ in fourteen single donor HLM showed that DCA 1β- and 5β-hydroxylation had better correlation with MDZ and T oxidation. The difference in correlation can be partially explained by metabolic phenotype. Data obtained in this work provided robust evidence that DCA 1β- hydroxylation and DCA 5β-hydroxylation can be used as *in vitro* index reactions for CYP3A activities.

**Materials and Methods**

**Materials and Reagents**

DCA, GDCA, and 6β-hydroxytestosterone (6βh-T) were purchased from Sigma-Aldrich (St. Louis, MO). 1βh-DCA was purchased from QCC (Newark, DE). MDZ, 1'-hydroxymidazolam (1'h-MDZ), and 4-hydroxymidazolam (4h-MDZ) were obtained from Cerilliant (Austin, Texas), MedChemExpress (Monmouth Junction, NJ), Carbosynth (Berkshire, UK), respectively. T and progesterone (PROG, internal standard used for the quantification of T and 6βh-T) were purchased from Nine-Dinn Chemistry (Shanghai, China). Clozapine (CLZ, internal standard used for the quantification of MDZ and its metabolites), and glycoursodeoxycholic acid-D4 (GUDCA-2,2,4,4-D4, internal standard used for the quantification of DCA, GDCA, and their metabolites) were procured from Sigma-Aldrich and IsoSciences (Ambler, PA), respectively. NADPH regenerating system solution A (NADPH-A) contained 66 mM glucose-6-phosphate, 26 mM NADP⁺, and 66 mM MgCl₂ in H₂O, NADPH regenerating system solution B (NADPH-B) contained 40 U/mL glucose-6-phosphate dehydrogenase in 5 mM sodium citrate. 0.5 M pH 7.4 phosphate buffer saline (PBS), NADPH-A, and NADPH-B were obtained from Corning (Tewksbury, MA). LC-MS grade acetonitrile, methanol, and formic acid were procured from Sigma-Aldrich. Dimethylsulfoxide (DMSO) of HPLC grade, was obtained from Thermo Fisher Scientific (Waltham, MA, USA). Ultra-pure water was obtained by employing a Milli-Q system (Millipore, Bedford).

**Synthesis of 5βh-DCA**

5βh-DCA was synthesized from DCA using the method described in reference (Iida et al., 2001) and our recent report (Zhang et al., 2019). The ¹H nuclear magnetic resonance (¹H-NMR) spectrum of 5βh-DCA was shown in Figure S1. The following is the spectrum data for the 5βh-DCA. ¹H NMR (400 MHz, Methanol-d4): δ 3.96 (d, 1H), 3.90 (m, 1H), 2.35 (m, 1H), 2.21 (m,
1H), 2.08 – 2.00 (m, 1H), 1.91 – 1.74 (m, 5H), 1.66 – 1.48 (m, 9H), 1.47 – 1.35 (m, 6H), 1.12 (m, 2H), 1.01 (d, 3H), 0.88 (s, 3H), 0.71 (s, 3H).

**Synthesis of 1βh-GDCA and 5βh-GDCA**

We synthesized 1βh-GDCA and 5βh-GDCA from 1βh-DCA and 5βh-DCA according to the procedure described in reference (Sepe et al., 2012) with some modifications. In brief, 1βh-DCA or 5βh-DCA (20 mg, 0.05 mmol) in dry N, N-Dimethylformamide (DMF) (1.2 mL) was treated with 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM) (40.6 mg, 0.15 mmol) and triethylamine (170 μL) and the mixture was stirred at room temperature for 10 min. Glycine ethyl ester (40.9 mg, 0.40 mmol) was then added to the mixture and stirring was continued for 24 h. Dissolve 5% lithium hydroxide in a mixed solution of tetrahydrofuran, methanol and water (5:3:2) then add it (4 mL) to the mixture. After stirring for 6 h at room temperature, the alkaline solution was adjusted to pH 2, loaded onto a C18 silica gel column. Elution with appropriate proportions of methanol and water gave the 1βh-GDCA (12 mg, 65% over two steps) or 5βh-GDCA (13 mg, 69% over two steps). ¹H-NMR spectra of 1βh-GDCA and 5βh-GDCA were summarized in Figure S2-S3. The following was the ¹H-NMR data of 1βh-GDCA: ¹H-NMR (400 MHz, Methanol-d₄) δ 4.04 – 3.96 (m, 1H), 3.95 (d, 1H), 3.86 (s, 2H), 3.82 (d, 1H), 2.32 (m, 1H), 2.17 (m, 1H), 1.93 – 1.68 (m, 9H), 1.67 – 1.54 (m, 4H), 1.53 – 1.38 (m, 6H), 1.30 (q, 3H), 1.02 (m, 6H), 0.72 (s, 3H). The following was the ¹H-NMR data of 5βh-GDCA: ¹H NMR (400 MHz, Methanol-d₄) δ 3.97 (d, 1H), 3.91 (d, 1H), 3.89 (s, 2H), 2.32 (m, 1H), 2.18 (m, 1H), 2.04 (t, 1H), 1.81 (m, 5H), 1.63 – 1.33 (m, 15H), 1.11 (m, 2H), 1.03 (d, 3H), 0.88 (s, 3H), 0.71 (s, 3H).

**Human Liver Microsomes and Recombinant CYP3A Enzymes**

The pooled HLM from 200 adult donors of mixed gender were purchased from XenoTech, LLC (Lenexa, KS). Human rP450s, which included CYP1A2, -1B1, -2A6, -2B6, -2C8, -2C9*1, -2C18, -2C19, -2D6*1, -3A4, -3A5, -3A7, -2E1, -2J2, -4A11, -4F2, -4F3B, and -4F12, were obtained from Corning (Tewksbury, MA). Fourteen single donor HLMs were acquired from BD Bioscience (Woburn, MA). Donor information provided by vendor was summarized in Table S1.

**In Vitro Activity Assay of DCA, GDCA, MDZ and T Oxidations**

*In vitro* metabolism assay was conducted as previously described (Zhang et al., 2019). In brief, stock solutions of DCA, GDCA, MDZ and T were prepared in DMSO. In the kinetic...
analysis of DCA and GDCA oxidation catalyzed by HLM or human rP450s, the initial substrate concentrations were 1-1000 μM for DCA, 30-1000 μM for GDCA. In the reaction phenotyping studies of DCA and GDCA catalyzed by human rP450s, the initial substrate concentrations were 50 μM for DCA and 100 μM for GDCA. In the correlation analysis of oxidation rate of DCA and GDCA with MDZ and T catalyzed by fourteen single donor HLMs, the initial substrate concentrations were 50 μM for DCA, 100 μM for GDCA, 4 μM for MDZ and 10 μM for T. In the kinetic analysis of DCA and MDZ in fourteen single donor HLMs, the initial substrate concentrations were 5-300 μM for DCA and 4-300 μM for MDZ.

The 100-μL incubation system of DCA or GDCA contained 2.5 μL liver microsomes (protein concentration of 20 mg/mL) or rCYP enzymes (2.0 nmol protein/mL), 5.0 μL NADPH-A, 1.0 μL NADPH-B, 1.0 μL working solution of substrate, and 90.5 μL 0.1 M PBS (pH 7.4). The MDZ incubation system contained 0.5 μL liver microsomes, 5.0 μL NADPH-A, 1.0 μL NADPH-B, 1.0 μL working solution of substrate, and 92.5 μL 0.1 M PBS (pH 7.4). The T incubation system contained 1.25 μL liver microsomes, 5.0 μL NADPH-A, 1.0 μL NADPH-B, 1.0 μL working solution of substrate, 91.75 μL 0.1 M PBS (pH 7.4). The protein concentrations in the incubation media differed depending on the substrate used. For DCA and GDCA, the final protein level was 50 pmol/mL of rP450s or 0.5 mg/mL of liver microsomes. For MDZ and T, the final protein level was 0.1 mg/mL and 0.25 mg/mL of liver microsomes, respectively. All samples were processed in triplicate, and the incubation system contained 1% (v/v) concentration of DMSO. Metabolism was initiated by adding NADPH-B after a 5-minute preincubation at 37°C in a shaking water bath. The reactions were quenched by adding ice-cold acetonitrile containing internal standards at 60 min for DCA, 120 min for GDCA, 10 min for MDZ, and 5 min for T. The proteins were removed by centrifugation at 12,000 rpm for 20 minutes at 4°C. The resulting supernatant, after appropriate dilution, was then analyzed using liquid chromatography with tandem mass spectrometry (LC-MS/MS).

Quantitative Determination of Metabolites in Incubation

Quantitative determinations were performed on ACQUITY UPLC coupled to Xevo TQS mass spectrometer (Waters, Milford, MA). The bioanalytical methods for each substrate and its metabolites were described in the supporting information. The calibration and quality control (QC) samples were prepared in 50% acetonitrile in water. The calibration ranges were 0.275 to 55 μM (DCA), 0.25 to 50 μM (1βh-DCA and 5βh-DCA), 0.05 to 10 μM (1βh-GDCA and 5βh-
GDCA), 0.006 to 6.0 μM (MDZ), 0.004 to 4.0 μM (1' h-MDZ and 4h-MDZ), 0.3 to 15 μM (T), 0.2 to 10 μM (6βh-T) with correlation coefficient $R^2 > 0.99$. The QC samples were allocated into each bioanalytical run. The concentrations of the QC samples were 0.825, 27.5, 41.25 μM for DCA, 0.75, 25, 37.5 μM for 1βh-DCA and 5βh-DCA, 0.15, 5.0, 7.5 for 1βh-GDCA and 5βh-GDCA, 0.018, 3.0, 4.5 μM for MDZ, 0.012, 2.0, 3.0 μM for 1'h-MDZ and 4h-MDZ, 0.45, 7.5, 11.25 μM for T, and 0.3, 5.0, 7.5 μM for 6βh-T. The relative errors of QC samples were -8.3% to 9.0% for DCA, -11.6% to 13.0% for 1βh-DCA, -13.1% to 12.3% for 5βh-DCA, -6.2% to 13.5% for 1βh-GDCA, -13.6% to 11.4% for 5βh-GDCA, -6.8% to 10.8% for 5βh-DCA, -5.1% to 3.1% for 1'h-MDZ, -10.1% to 3.8% for 1'h-MDZ, 1.0% to 12.7% for T, 1.8% to 8.7% for 6βh-T.

**Data Processing**

The LC-MS/MS raw data was processed using UNIFI (V1.8, Waters, Milford, MA, USA). The absolute metabolite formation rates were calculated in the unit of pmol/min/mg protein for incubations in HLMs, and nmol/min/nmol P450 for incubations in rP450s. In accordance with the previous report (Martinez et al., 2020), the absolute metabolite formation rates in rP450s were adjusted by multiplying the mean estimates of P450 abundance (pmole P450 per mg microsomal protein) (Couto et al., 2019), which were 14.14, 25.62, 6.78, 29.78, 37.53, 1.60, 3.43, 6.05, 54.38, 0.64, 28.52, 8.63, 5.54, 12.22, 5.06 for human CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 2J2, 3A4, 3A5, 3A7, 4F2, 4F12 in HLM. GraphPad Prism (V8.0.2, GraphPad Software, LaJolla, CA) were used for kinetic analysis, statistical analysis and graphic presentation. The kinetic parameters were determined by best fitting to models, including Allosteric sigmoidal (Hill), Michaelis-Menten (MM), or Substrate inhibition. The intrinsic clearance ($CL_{int}$) was calculated as the ratio of $V_{max}/K_m$ ($S_{50}$). To analyze the relationship between metabolite formation rates or $V_{max}$ and $CL_{int}$ of DCA and GDCA with MDZ and T, Pearson correlation analysis was conducted using data obtained from fourteen single donor microsomes.

**Results**

**1β- and 5β-hydroxylation kinetics of DCA and GDCA in HLM**

Table 1 lists the apparent kinetic data for the tested index reactions in HLM with the kinetic plots illustrated in Figure 2. A gradual loss of oxidation activities appeared at substrate levels higher than 400 μM for DCA and 250 μM for GDCA (Figure 2a-d). The abnormal kinetics was
not explained by the substrate inhibition model (Figure S4) and might be associated with the detergent effects of DCA and GDCA (Chen et al., 2019). A limited substrate concentration ranges was therefore selected for kinetics fitting of DCA (1-400 μM) and GDCA (30-250 μM), within which the metabolic turnover did not begin to decrease (Figure 2A-D). The apparent kinetics of all index reactions over the tested substrate ranges were best fit with Hill model. The Hill $R^2$ (goodness of fit) for most fittings was $> 0.98$. The Hill coefficients for all reactions were $>1$, indicating a positive cooperativity. The $S_{50}$ values of DCA 1β-hydroxylation (116 μM) and 5β-hydroxylation (149 μM) were lower than those for GDCA 1β-hydroxylation (210 μM) and 5β-hydroxylation (216 μM), respectively. The $CL_{int}$ values decreased in the following order, DCA 1β-hydroxylation, DCA 5β-hydroxylation, GDCA 1β-hydroxylation, and GDCA 5β-hydroxylation. According to the $CL_{int}$ data, HLM showed the strongest activity to DCA 1β-hydroxylation and the weakest activity to GDCA 5β-hydroxylation.

**1β- and 5β-hydroxylation phenotyping for DCA and GDCA**

Eighteen rP450s were evaluated for their selectivity and activity toward the tested index reactions, using appropriate substrate levels based on their apparent kinetics in HLM. Figure 3A showed the absolute formation rate of probe metabolites in human rP450s. CYP3A4 and CYP3A7 exhibited the highest catalytic activity for all oxidation reactions, while CYP3A5 showed significantly lower activity, consistent with previous data obtained with the enzyme-digestion technique (Zhang et al., 2019). Both CYP3A4 and CYP3A7 showed the greatest activity toward DCA 1β-hydroxylation and the least activity toward GDCA 5β-hydroxylation. The absolute activities of these index reactions in human rP450s were adjusted according to the absolute P450 abundance estimates in HLM (Couto et al., 2019) (Figure 3B). As a result of the higher abundance of CYP3A4 in HLM, the adjusted CYP3A4 activity increased, while those of CYP3A5 and 3A7 decreased compared with their absolute activities. According to the adjusted data, the percentage contributions of the reactive human rP450s were illustrated for each index reaction (Figure 3C). The specific metabolite formation rate data as well as the adjusted formation rate based on the P450 abundance estimates from Cuoto et al. 2019 were summarized in Table S2. The results showed that 1β- and 5β-hydroxylation of DCA and GDCA had the most contribution from CYP3A4 and 3A7, DCA 5β-hydroxylation showed the greatest selectivity for CYP3A4, whereas GDCA 1β-hydroxylation had equivalent contributions from CYP3A4 and CYP3A7.
1β- and 5β-hydroxylation kinetics of DCA and GDCA in CYP3A4 and CYP3A7

According to the phenotyping data of human rP450s, the kinetic data were comparably acquired in the recombinant CYP3A4 and CYP3A7. Table 2 and Figure 4 listed the kinetic parameters and the kinetic plots of DCA and GDCA in CYP3A4 and CYP3A7. A gradual loss of oxidation activities also appeared in CYP3A4 and CYP3A7 (Figure 4a-d), which also could not be explained by the substrate inhibition model (Figure S5-S6). The substrate ranges of DCA (1-300 μM) and GDCA (30-200 μM) were selected for nonlinear fitting with the Hill model (Figure 4A-D). The Hill R² for all fittings was >0.98. The Hill coefficients of DCA 1/5β-hydroxylation, GDCA 5β-hydroxylation in CYP3A4, DCA 1β-hydroxylation in CYP3A7 were >1, while the other reactions were <1, indicating a positive and no or negative cooperativity, respectively. According to the CL_int data, the catalytic activity of CYP3A4 decreased in turn for DCA 1β-hydroxylation, DCA 5β-hydroxylation, GDCA 1β-hydroxylation, and GDCA 5β-hydroxylation, and CYP3A7 catalytic activity gradually decreased for DCA 1β-hydroxylation, GDCA 1β-hydroxylation, DCA 5β-hydroxylation, GDCA 5β-hydroxylation. Consistent with the activity data acquired in HLM, the metabolic turnover of DCA 1β- and 5β-hydroxylation was much higher than that of GDCA. The CL_int ratio of CYP3A4 to CYP3A7 gradually decreased for GDCA 5β-hydroxylation (2.00), DCA 5β-hydroxylation (1.33), DCA 1β-hydroxylation (0.60), and GDCA 1β-hydroxylation (0.14), which indicated that 5β-hydroxylation of DCA and GDCA was more CYP3A4 specific.

DCA oxidation is more correlated to CYP3A index reactions than GDCA oxidation

Metabolite formation rates of DCA (50 μM) and GDCA (100 μM) were analyzed in a panel of HLMs from 14 single adult donors in comparison to MDZ 1’- and 4- hydroxylation and T 6β-hydroxylation (Table S3). According to the metabolite formation data, DCA and GDCA showed much lower metabolic turnover than both MDZ and T. Pearson correlation analysis between the activities of tested reactions was shown in Figure 5. It was disclosed that both MDZ 1’- and 4-hydroxylation correlated well to T 6β-hydroxylation. Compared to GDCA oxidations, DCA oxidations were better correlated with the oxidation of both MDZ and T, which might be partly associated with the higher oxidation activity of DCA than GDCA. Among the tested reactions, GDCA 1β-hydroxylation showed none correlation to the oxidation of both MDZ and T, while the oxidation of either MDZ or T was significantly correlated with DCA 1β-hydroxylation, DCA 5β-
hydroxylation and GDCA 5β-hydroxylation, in which DCA 5β-hydroxylation showed the best correlation coefficient.

**DCA oxidations correlated well to midazolam oxidation in individual liver microsomes**

Based on the correlation data of reactions at single substrate level, the correlation of oxidation activities between DCA and MDZ were further confirmed at substrate ranges of 5-300 μM and 4-300 μM, respectively. Table S4 listed the apparent kinetic data of DCA oxidation in fourteen single donor microsomes with the kinetic plots illustrated in Figure S7. Similarly, the kinetics of DCA in fourteen single donor microsomes were best fit with Hill model with the Hill coefficients >1. The kinetic parameters and the kinetic plots of MDZ oxidation in fourteen single donor microsomes were shown in Table S5 and Figure S8, in which MDZ 1’- and 4-hydroxylation were best fit with the substrate inhibition model. According to the average CL_{int} data in the tested microsomes, the activities of reactions decreased in turn for MDZ 1’-hydroxylation (336 μL/min/mg protein), MDZ 4-hydroxylation (8.03 μL/min/mg protein), DCA 1β-hydroxylation (1.55 μL/min/mg protein) and DCA 5β-hydroxylation (0.151 μL/min/mg protein). indicated that DCA had much lower metabolic turnover than MDZ. As shown in Figure 6, the CL_{int} data of both DCA 1β-hydroxylation and DCA 5β-hydroxylation correlated well with that of either MDZ 1’-hydroxylation or MDZ 4-hydroxylation, with the best correlation appeared between the CL_{int} of DCA 5β-hydroxylation and MDZ 4-hydroxylation (r=0.9538, P< 0.0001).

**Discussion**

Chenodeoxycholic acid (CDCA) and CA are the primary BAs synthesized from cholesterol in human (Russell, 2003; de Aguiar Vallim et al., 2013). In the intestine, CA and CDCA undergo 7-dehydroxylation by gut microbiota to form secondary BAs, DCA and lithocholic acid (LCA). These secondary BAs then circulate back to the liver through the enterohepatic circulation (Hofmann, 2009). The secondary BAs are exogenous to the host with strong lipophilicity, cell permeability, and toxicity (Hofmann, 2004; Jia et al., 2018), raising the question of what enzymes are involved in the tertiary metabolism of secondary BAs (Araya and Wikvall, 1999; Bodin et al., 2005; Hayes et al., 2016). We have recently disclosed that human CYP3A4 and CYP3A7 are exclusively responsible for the tertiary oxidations of DCA, GDCA, and TDCA regioselectivity at C-1β, C-6α, C-5β, C-4β, C-6β, and C-19 (Zhang et al., 2019). Accordingly, the tertiary metabolism of secondary BAs may be regarded as the host's response to stress from
gut microbiome derived xenobiotics. Among the tertiary metabolites of DCA, the C-1β and C-5β oxidized products seem to exist as 1βh-GDCA and 5βh-GDCA without significant glucuronidation and/or sulfation (Zhang et al., 2019). The in vitro 1β- and 5β-hydroxylation metabolism of DCA and GDCA were therefore compared in this work to investigate the disposition pathway of them. Based on the kinetic data in both HLM and rP450s, the metabolic turnover of DCA 1β- and 5β-oxidation was found to be 5-10 times higher than that of GDCA. This difference is related to either the binding affinity of DCA and GDCA to CYP3A substrate pocket or the allosteric effect during the redox circle of CYP3A. It was accordingly concluded that 1βh-GDCA and 5βh-GDCA come from the conjugation of 1βh-DCA and 5βh-DCA rather than oxidation of GDCA.

The prerequisite for an index reaction is that it is selectively catalyzed by the enzyme of interest (Hayes et al., 2016). The phenotyping data clearly showed that DCA and GDCA 1β- and 5β-hydroxylation were predominantly catalyzed by CYP3A4 and CYP3A7 (>98%). DCA 1β-hydroxylation, DCA 5β-hydroxylation and GDCA 5β-hydroxylation had more contributions from CYP3A4 (>80%), while GDCA 1β-hydroxylation had equivalent contributions from CYP3A4 (41.04%) and CYP3A7 (57.97%). According to our recently reported phenotyping data of MDZ and T (Wu et al., 2022), the contributions of CYP3A4, CYP3A5 and CYP3A7 were 65.18%, 28.47% and 2.07% for the MDZ 1’-hydroxylation, 32.67%, 32.67% and 49.61% for MDZ 4- hydroxylation, and 91.39%, 6.08% and 2.22% for the T 6β-hydroxylation reaction, respectively. Accordingly, DCA 1β-hydroxylation, DCA 5β-hydroxylation and GDCA 5β-hydroxylation correlated well to the oxidation of either MDZ or T in single donor microsomes, while GDCA 1β-hydroxylation showed no correlation. The difference in cross-correlation between the tested reactions may be partially explained by metabolic phenotype particularly associated with the contribution from CYP3A7, which is also expressed in adult livers (Burk et al., 2002; Sim et al., 2005; Couto et al., 2019). To this end, it is concluded that DCA 1β-hydroxylation, DCA 5β-hydroxylation and GDCA 5β-hydroxylation exhibit similar metabolic phenotype to T 6β-hydroxylation.

The assessment of in vitro CYP3A activity exhibits significant substrate-dependent phenomenon and often results in varying outcomes with different probes. Currently, MDZ 1’-hydroxylation and T 6β-hydroxylation are widely employed as two complementary index reactions to assess the in vitro activity of CYP3A. According to the phenotyping data, T and
MDZ seem to belong to two distinct classes of CYP3A substrates, with T 6β-hydroxylation as indicator mainly of CYP3A4, MDZ 1’-hydroxylation as indicator of both CYP3A4 and CYP3A5, and MDZ 4- hydroxylation as indicator of CYP3A4, CYP3A5 and CYP3A7. T is metabolized in HLM mainly into 6βh-T, 2β-hydroxytestosterone, 15β-hydroxytestosterone, 4-hydroxyandrostenedione, and androstenedione (Usmani et al., 2003; Niwa et al., 2015), in which androstenedione and 6β-T accounted for about 55% and 37% of the degraded substrate, respectively (Wu et al., 2022). As the major side reaction of T 6β-hydroxylation, the interconversion of androstenedione and T is catalyzed by HSD17B in HLM and accordingly brings inevitable bias for the substrate level. In comparison, the substrate level of DCA in HLM are much less affected by side reactions. On the other hand, androgens are regulated substances because their exposure is harmful to the human body. In comparison, DCA and GDCA are more easily accessible without appreciated harms to human. Based on the fact that DCA oxidation exhibits strong correlation with either MDZ or T, we propose that DCA may serve as an alternative of T for CYP3A index reactions.

A limitation of using DCA oxidation as CYP3A index reactions lies in its lower activity than MDZ and T oxidation. According to the metabolite formation data at single substrate level in single donor HLMs, MDZ 1’-hydroxylation and T 6β-hydroxylation showed 2.5-45 folds and 1.8-10 folds higher activities than DCA 1β-hydroxylation (Table S3), which had the strongest activity among the tested reactions of DCA and GDCA in this work. Since the $S_{50}$ of DCA is much higher than that of MDZ, the $CL_{int}$ of MDZ 1’-hydroxylation was 93-344 folds higher than that of DCA 1β-hydroxylation in single donor HLMs (Table S4-S5). The lower turnover results in formation of less product requiring higher protein concentrations, longer incubation times, and more sensitive analytical methods when using DCA oxidation as CYP3A index reaction. Moreover, the oxidation of DCA and GDCA is characterized by the gradual loss of reactivity at substrate levels higher than 400 μM for DCA and 250 μM for GDCA. The underlying mechanism is closely associated with the unique amphipathic properties of BAs. A summary of our exploration of this phenomenon will be provided in the near future.

Assessment of the in vivo activities of CYP3A has been reported to be performed by administration of exogenous probe substrates, such as MDZ (Gorski et al., 1994; Patki et al., 2003) and erythromycin (Gonzalez, 1990; Rivory et al., 2001). As the substrate of CYP3A4 and CYP3A5, MDZ oral clearance is the gold standard for assessing the in vivo activities of
CYP3A4/5. The erythromycin breath test employs the intravenous administration of $^{14}$C-erythromycin followed by the measurement of $^{14}$CO$_2$ in the breath (Rivory et al., 2001). Both methods bring xenobiotics into human body. Although erythromycin breath test is less invasive, it produced inconsistent results compared to the MDZ method due to without accounting for intestinal CYP3A activities (Kinirons et al., 1999). Obviously, the CYP3A catalyzed endogenous reactions may serve as none-invasive CYP3A probes. The urine 6βh-cortisol/cortisol ratio is the first proposed endogenous CYP3A probe (Ged et al., 1989) with acceptable correlation with MDZ clearance (Shin et al., 2016). However, the probe of 6βh-cortisol/cortisol inevitably encounters physiological bias because the glucocorticoid metabolism is vulnerable to stress, infection and circadian rhythm (Galteau and Shamsa, 2003). The plasma 4β-hydroxycholesterol is another endogenous CYP3A probe (Bodin et al., 2001). However, 4β-hydroxycholesterol is not sensitive to evaluate the short-term CYP3A inhibition status due to a long half-life (Diczfalusy et al., 2011; Kasichayanula et al., 2014). Novel endogenous probes are still the unmet need to phenotype the in vivo activity of CYP3A.

CYP3A enzymes are expressed not only in the liver but also on the intestinal mucosa, where they contribute considerably to first-pass metabolism of drugs (Lin and Lu, 2001; Paine et al., 2006). DCA is a secondary bile acid synthesized by bacteria in the gut and recovered into the liver through enterohepatic circulation. Our previous in vitro studies had demonstrated that the metabolic turnover of DCA in human intestinal microsomes is approximately one-third compared to that in HLM (Table S6). It had also been evidenced that the serum ratios of 1βh-DCA/DCA or 5βh-DCA/DCA are predictive of the in vivo CYP3A activities indicated by the oral MDZ clearance in beagle dogs (Zeng et al., 2021). Hence, DCA oxidation shows promise to reflect the in vivo enterohepatic CYP3A activity compared to 6βh-cortisol/cortisol and 4β-hydroxycholesterol. However, the dynamic range of DCA oxidation appeared narrower than MDZ clearance in dogs after treatment with ketoconazole, which might be attributed to the relatively lower CYP3A catalyzed oxidation activities of DCA than MDZ. Given that 1βh-GDCA and 5βh-GDCA are circulated tertiary BAs in human, it is proposed that the ratios of 1βh-GDCA/DCA or 5βh-GDCA/DCA might serve as a potential in vivo probe of CYP3A, because 1βh-GDCA and 5βh-GDCA come from DCA oxidation followed by conjugation. However, the studies of tertiary metabolism of DCA and GDCA in human are still quite limited. It remains unclear the baseline levels of 1βh-DCA, 5βh-DCA, 1βh-GDCA, 5βh-GDCA in
contrast to DCA and GDCA in plasma and urine. We are engaged in such fundamental study to ascertain their baseline levels and daily excretion and lay a foundation to verify this hypothesis.

In conclusion, this work clarified that DCA 1β- and 5β-hydroxylation may serve as in vitro index reactions for CYP3A activities. In vitro evidence was also provided for the metabolic pathway to synthesize the conjugated tertiary BAs in humans. 1βh-GDCA and 5βh-GDCA in the human body are formed through the conjugation of 1βh-DCA and 5βh-DCA rather than the oxidation of GDCA. Correlation analysis showed that DCA 5β-hydroxylation exhibited the strongest correlation with MDZ oxidation, while DCA 1β-hydroxylation displayed the highest metabolic turnover. Considering the similarities in metabolic phenotypes between DCA 1β/5β-hydroxylation and T 6β-hydroxylation, the presence of more side reactions of T in HLM, and the disadvantages of T in labor protection and occupational health, it is suggested that DCA 1β- and 5β-hydroxylation are alternative to T 6β-hydroxylation as in vitro CYP3A index reactions.
Acknowledgements

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Data Availability Statement

The authors declare that all the data supporting the findings of this study are available within the paper and its Supplemental Data.

Authorship Contributions

Participated in research design: Wang C, Xu L, Miao J, Lan K
Performed data analysis: Wang C, Miao J, Lan K
Wrote or contributed to the writing of the manuscript: Wang C, Cheng B, Miao J, Lan K

References


Footnotes

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b) *contributed equally to this work.

c) The authors declare no conflict of interest.
Legends for Figures

**Figure 1.** Theoretical oxidation and conjugation metabolic pathways of DCA in the liver

**Figure 2.** Kinetic plots 1β-hydroxydeoxycholic acid (1βh-DCA), 5β-hydroxydeoxycholic acid (5βh-DCA), 1β- hydroxyglycodeoxycholic acid (1βh-GDCA), and 5β-hydroxyglycodeoxycholic acid (5βh-GDCA) formation by human liver microsomes (HLM). Formation rate was plotted as a function of substrate concentration for 1-1000 µM (a, b) or 1-400 µM (A, B) deoxycholic acid (DCA, 60 min), 30-1000 µM (c, d) or 30-250 µM (C, D) glycodeoxycholic acid (GDCA, 120 min) with HLM (0.5 mg/mL). Lines represented nonlinear regression curves. Data was shown as mean ± SD of triplicates.

**Figure 3.** Reaction phenotyping results of DCA (50 µM), GDCA (100 µM) in the recombinant human CYP enzymes (rCYP, 50 pmole/mL). The absolute metabolite formation rates of human rCYP enzymes (A); the adjusted metabolite formation rates based on the published absolute CYP abundance estimates in HLM (B); the pie chart for the relative contributions of active P450 isoforms to the tested reactions (C). Data was shown as mean ± SD of triplicate values.

**Figure 4.** Kinetic plots of 1βh-DCA, 5βh-DCA, 1βh-GDCA, and 5βh-GDCA formation by CYP3A4 and CYP3A7. Formation rate was plotted as a function of substrate concentration for 1-1000 µM (a, b) or 1-300 µM (A, B) DCA (60 min), 30-1000 µM (c, d) or 30-200 µM (C, D) GDCA (120 min) with CYP3A4 and CYP3A7 (0.5 mg/mL). Lines represented nonlinear regression curves. Data was shown as mean ± SD of triplicates. Formation rate was plotted as a function of substrate concentration for 1-300 µM DCA (60 min), 30-200 µM GDCA (120 min) with CYP3A4 and CYP3A7 (0.5 mg/mL). Lines represented nonlinear regression curves. Data was shown as mean ± SD of triplicates.

**Figure 5.** Correlation analysis of 1βh-DCA, 5βh-DCA, 1βh-GDCA, and 5βh-GDCA formation with 1’-hydroxymidazolam (1’h-MDZ), 4-hydroxymidazolam (4h-MDZ), and 6β-hydroxytestosterone (6βh-T) formation in fourteen single donor microsomes (HFC205, HFC208, HFH617, HFH705, HG18, HG43, HG43-1, HG64, HH13-2, HH37, HH519, HH581, HH741, HH837). Pearson correlation analysis was conducted between the oxidation of DCA (50 µM, 60 min) and GDCA (100 µM, 120 min) with MDZ (4 µM, 10 min) and T (10 µM, 5 min). The
concentrations of protein were 0.5 mg/mL for DCA and GDCA, 0.1 mg/mL for MDZ, and 0.25 mg/mL for T.

**Figure 6.** Correlation analysis of 1βh-DCA and 5βh-DCA formation with 1’h-MDZ and 4h-MDZ in fourteen single donor microsomes (HFC205, HFC208, HFH617, HFH705, HG18, HG43, HG43-1, HG64, HH13-2, HH37, HH519, HH581, HH741, HH837). Pearson correlation analysis was conducted between the kinetic parameters (CL\text{int}) of DCA (50 μM, 60 min) with MDZ (4 μM, 10 min) oxidation. The concentrations of protein were 0.5 mg/mL for DCA and 0.1 mg/mL for MDZ.
Table 1. Kinetic parameters for the hydroxylation of deoxycholate (DCA), and glycodeoxycholic acid (GDCA) in the pooled human liver microsomes (HLM).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>DCA</th>
<th>GDCA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tested range (μM)</td>
<td>1-400</td>
<td>30-250</td>
</tr>
<tr>
<td>HLM protein level (mg/mL)</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Incubation time (min)</td>
<td>60</td>
<td>120</td>
</tr>
<tr>
<td>Hydroxylation site</td>
<td>1β- 5β-</td>
<td>1β- 5β-</td>
</tr>
<tr>
<td>Best Fit to Model a</td>
<td>Hill</td>
<td>Hill</td>
</tr>
<tr>
<td>$V_{\text{max}}$ (pmol/min/mg protein)</td>
<td>203.7</td>
<td>28.5</td>
</tr>
<tr>
<td>$S_{50}$ or $K_{m}$ (μM)</td>
<td>116.5</td>
<td>149.1</td>
</tr>
<tr>
<td>$\text{CL}_{\text{int}} ^b$ (μL/min per mg protein)</td>
<td>1.75</td>
<td>0.19</td>
</tr>
<tr>
<td>Hill coefficient</td>
<td>1.50</td>
<td>1.43</td>
</tr>
<tr>
<td>Goodness of fit ($R^2$)</td>
<td>0.996</td>
<td>0.995</td>
</tr>
</tbody>
</table>

a: The kinetic parameters were determined by best fitting to models, including Michaelis-Menten (MM), Allosteric sigmoidal (Hill) or Substrate inhibition (SI).

b: The intrinsic clearance ($\text{CL}_{\text{int}}$) was calculated by the ratio of $V_{\text{max}}/K_{m}$ ($S_{50}$).
**Table 2.** Kinetic parameters for the hydroxylation of deoxycholate (DCA), and glycodeoxycholic acid (GDCA) in the recombinant human CYP3A4, and 3A7 at the protein levels of 50 pmole/mL.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Tested range (μM)</th>
<th>Incubation time (min)</th>
<th>Hydroxylation site</th>
<th>Best Fit to Model</th>
<th>( V_{\text{max}} ) (nmol/min/nmol P450)</th>
<th>( S_{50} ) or ( K_m ) (μM)</th>
<th>( \text{CL}_{\text{int}} ) (μL/min per nmol P450)</th>
<th>( \text{Hill coefficient} )</th>
<th>Goodness of fit (( R^2 ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCA</td>
<td>1-300</td>
<td>60</td>
<td>1( \beta )-</td>
<td>CYP3A4</td>
<td>6.1</td>
<td>93.8</td>
<td>0.065</td>
<td>0.991</td>
<td>0.994</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5( \beta )-</td>
<td>CYP3A7</td>
<td>0.4</td>
<td>113.8</td>
<td>0.003</td>
<td>0.990</td>
<td>0.989</td>
</tr>
<tr>
<td>GDCA</td>
<td>30-200</td>
<td>120</td>
<td>1( \beta )-</td>
<td>CYP3A4</td>
<td>0.3</td>
<td>188.7</td>
<td>0.004</td>
<td>0.993</td>
<td>0.983</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5( \beta )-</td>
<td>CYP3A7</td>
<td>3.3</td>
<td>308.3</td>
<td>0.001</td>
<td>0.985</td>
<td>0.975</td>
</tr>
</tbody>
</table>

\( a \): The kinetic parameters were determined by best fitting to models, including Michaelis-Menten (MM), Allosteric sigmoidal (Hill) or Substrate inhibition (SI).

\( b \): The intrinsic clearance (\( \text{CL}_{\text{int}} \)) was calculated by the ratio of \( V_{\text{max}}/K_m (S_{50}) \).
Figure 1
Figure 2

Metabolites formation (pmol/min/mg HLM)

1βh-DCA

5βh-DCA

1βh-GDCA

5βh-GDCA

[DCA] (μM) [DCA] (μM) [GDCA] (μM) [GDCA] (μM)

1βh-DCA 5βh-DCA 1βh-GDCA 5βh-GDCA

A B C D
Figure 3
Figure 4

- **A** 1βh-DCA
- **B** 5βh-DCA
- **C** 1βh-GDCA
- **D** 5βh-GDCA

**Metabolites formation (nmol/min/nmol CYP)**

**[DCA] (μM)**: 0 75 150 225 300

**[GDCA] (μM)**: 0 75 150 225 300

**[DCA] (μM)**: 0 100 200 300

**[GDCA] (μM)**: 0 100 200 300

**CYP3A4**

**CYP3A7**
Figure 5
Figure 6

![Graph showing correlations]

CL\textsubscript{int} of DCA (\mu L/min/mg protein)

CL\textsubscript{int} of MDZ (\mu L/min/mg protein)

1h-MDZ

4h-MDZ

1βh-DCA

5βh-DCA

r = 0.8186
P = 0.0003

r = 0.8852
P < 0.0001

r = 0.9023
P < 0.0001

r = 0.9538
P < 0.0001

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