***In vivo* cytochrome P450 activity alterations in diabetic nonalcoholic steatohepatitis mice**

Hui Li¹, John D. Clarke¹, Anika L. Dzierlenga¹, John Bear², Michael J. Goedken³ and Nathan J. Cherrington¹

¹University of Arizona, Department of Pharmacology and Toxicology; ²Univeristy of Arizona, Statistical Consulting Lab, Tucson, AZ 85721, USA; ³Rutgers University, Translational Sciences, Research Pathology Services, New Brunswick, NJ 08854, US

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Corresponding author:

Nathan J. Cherrington

1703 E. Mabel Street

Tucson, AZ 85721

Phone: 520 626-0219

Fax: 520 626-2466

Email: cherrington@pharmacy.arizona.edu

Nonalcoholic steatohepatitis (NASH) has been identified as a source of significant inter-individual variation in drug metabolism. A previous *ex vivo* study demonstrated significant changes in hepatic CYP activity in human NASH. This study evaluated the *in vivo* activities of multiple CYP isoforms simultaneously in prominent diabetic NASH mouse models. The pharmacokinetics of CYP selective substrates: caffeine, losartan and omeprazole changed significantly in a diabetic NASH mouse model, indicating attenuation of the activity of Cyp1a2 and Cyp2c29, respectively. Decreased mRNA expression of Cyp1a2 and Cyp2c29, as well as overall decrease in CYP protein expression was found in the diabetic NASH mice. Overall, these data suggest that the diabetic NASH model only partially recapitulates the human *ex vivo* CYP alteration pattern. Therefore, *in vivo* determination of the effects of NASH on CYP activity should be conducted in human, and more appropriate models are required for future drug metabolism studies in NASH.

Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease in the United States with a prevalence of 24% in adults (1) and 10%~20% in children (2). NAFLD encompasses a spectrum of liver manifestations ranging from simple steatosis to non-alcoholic steatohepatitis (NASH) (3), which may progress to cirrhosis and hepatocellular carcinoma (4, 5). NAFLD is commonly referred to as the hepatic manifestation of the metabolic syndrome (6, 7), because of its association with risk factors such as central obesity, diabetes mellitus, and dyslipidemia (8, 9). It is estimated that the prevalence of NAFLD among type 2 diabetics is as high as 69% (9), and the estimated prevalence of diabetes among NAFLD and NASH patients is 22.51% and 43.63%, respectively (1), suggesting that NAFLD/ NASH and diabetes are highly associated.

NASH has been identified as a potential contributing factor to variable drug response (VDR). It has been demonstrated that NASH can alter the metabolism and disposition of many drugs, including ezetimibe, pravastatin, methotrexate, morphine, acetaminophen and metformin (10–15). Remodeling of the hepatic absorption, distribution, metabolism, and excretion (ADME) pathways has been identified as the general mechanism for these changes, which can exacerbate the adverse drug reactions associated with these drugs (12). The vast majority of altered ADME gene expression changes in NAFLD were observed after the progression to NASH, suggesting that NASH is where VDR will be most likely (16). Previously, our group has shown that *ex vivo* activities of CYPs are altered in human NASH liver (17).

Various experimental models have been established to mimic the pathological conditions of human NASH, although to date there is no single animal model that can accurately encompass

the full spectrum of human NASH progression (18). The leptin deficient *ob/ob* mice are widely used as a model of obesity, type II diabetes, and NAFLD, as they develop steatosis spontaneously (19, 20). In addition, they exhibit metabolic signatures including insulin resistance, hyperinsulinemia, hyperglycemia and hyperlipidemia (21). However, additional stimuli are required to drive the pathological development of NASH features, such as inflammation and fibrosis (22). The methionine- and choline- deficient (MCD) diet, which disrupts the hepatic β -oxidation and the production of very low-density lipoprotein (VLDL) in animals, is widely employed to induce NASH in animal models (18, 23). The *ob/ob* MCD model develops mRNA and protein expression profiles of hepatic transporters that are consistent with human NASH (24), and was employed as a model for NASH in several mechanistic studies (15, 25, 26). A 4-week MCD diet was fed to *ob/ob* mice to create our experimental diabetic NASH model.

In vivo analysis of CYP activity is the most accurate way to assess CYP function (27). Administration of probe drugs in a cocktail approach is a preferable method to analyze *in vivo* activity of multiple CYP isoforms simultaneously (28). Probe drugs used in this study include caffeine, losartan, omeprazole, midazolam and dextromethorphan, which are the selective substrates of human CYP1A2, CYP2C9, CYP2C19, CYP3A4 and CYP2D6 isoforms respectively. In the present study, we characterized the reported mouse orthologs, or highly potential counterparts of human CYP isoforms listed above: Cyp1a2, Cyp2c29, Cyp2d22 and Cyp3a11, in order to reflect NASH-associated changes in CYP activity in mouse models (29–32).

The purpose of the present study was to determine the *in vivo* alterations of the major CYP enzymatic activities in *ob/ob* and *ob/ob* MCD mouse models. By comparing these data to a previous *ex vivo* human study (17), we determined whether this model can accurately replicate the CYP remodeling seen in human NASH.

Material and Methods

Chemicals and Reagents:

Caffeine, losartan, omeprazole, midazolam, 1'-hydroxymidazolam, phenacetin, dextromethorphan and dextrophan were purchased from Sigma-Aldrich (St Louis, MO). Paraxanthine, E3174 (losartan carboxylic acid) and 5-hydroxyomeprazole were purchased from Toronto Research Chemicals. All other chemicals were obtained from commercial sources.

Animals:

C57BL/6J wild type (WT) and leptin-deficient (*ob/ob*) male mice were purchased from Jackson Laboratory (Bar Harbor, ME) at 8 weeks of age (n=5 for each group) and housed on a 12-hour light and 12-hour dark cycle in the University of Arizona animal care facility. The animal studies were approved by the University of Arizona Animal Care and Use Committee. The mice in WT and *ob/ob* groups were fed a methionine and choline resupplemented diet (Dyets Inc. Bethlehem, PA) for 4 weeks, while the mice in the *ob/ob* MCD group were fed a MCD diet for 4 weeks to induce NASH (Dyets Inc. Bethlehem, PA).

Histopathological analysis and plasma chemistries:

Formalin-fixed and paraffin embedded liver tissue were stained with hematoxylin and eosin (H&E) and pathology scored by a veterinary pathologist. Liver tissues were scored for steatosis, necrosis, inflammation, fibrosis and biliary hyperplasia. Pathology scores were as follows: 0, no significant lesions (0%); 1, minimal (<10%); 2, mild (10~25%); 3, moderate (25~40%); 4, marked (40~50%); 5, severe (>50%). Whole blood was centrifuged for 15 minutes at 5000 rpm, 4°C to get plasma. Plasma collected prior to gavage was used to quantify peripheral blood

insulin and glucose concentrations. Insulin was measured using an ELISA kit (Millipore, Billerica, MA) and glucose was measured using an absorbance-based assay kit (Abcam, Cambridge, MA), according to the manufactures' protocol.

In Vivo pharmacokinetics of probe drugs:

Mice were dosed by oral gavage with five-drug cocktail comprising caffeine (5mg/kg), losartan (40mg/kg), omeprazole (15mg/kg), dextromethorphan (20mg/kg) and midazolam (5mg/kg). In all cases, whole blood (30 μ L) was taken from the tail vein at intervals of drug post-administration (30, 60, 120, 240 and 360minutes), into a tube coated with heparin. Plasma samples were obtained by centrifugation.

Sample preparation and LC-MS/MS analysis:

The plasma samples (5 μ L) were spiked with an internal standard (10 μ L of 400 ng/mL Phenacetin), and mixed with 100 μ L of acetonitrile for protein precipitation. Chromatographic separation was achieved using a gradient system of 0.1% (v/v) formic acid in water and 0.1%(v/v) formic acid in acetonitrile as follows: The HPLC column is a Luna C-18 (2) 3 μ 4.6x100mm (Phenomenex, Torrance, CA) with a C-18 guard cartridge 3*4mm. Analysis was performed on a TSQ Quantum Ultra triple quadrupole mass spectrometer with Surveyor MS pump plus and Surveyor Antosampler plus (Thermo Electron, San Jose, CA). The mass spectrometer was operated in electrospray positive ionization mode with a spray voltage of 4900V, sheath gas (nitrogen) flow rate of 49 arbitrary units and auxiliary gas flow rate of 5 arbitrary units. The capillary temperature is 320C. Argon was used as the collision gas at a pressure of 0.8 mtorr. The mass transitions were as follows: m/z 195 $\rightarrow$ 138 for Caffeine, m/z 181 $\rightarrow$ 124 for Paraxanthine, m/z 346 $\rightarrow$ 198 Omeprazole, m/z 362 $\rightarrow$ 214 for 5-hydroxyomeprazole, m/z 326 $\rightarrow$ 291 for

Midazolam, m/z 342→324 for 1-hydroxymidazolam, m/z 423→207 for Losartan, m/z 437→207 for EXP-3174, m/z 272→215 for Dextromethorphan, m/z 258→157 for Dextrorphan and m/z 180→110 for Phenacetin.

RNA isolation and mRNA analysis:

Total RNA was extracted from liver tissues using RNazol B reagent from Tel-Test Inc. (Friendswood, TX) according to the manufacture's protocol. Equal amounts of RNA (1µg) were used for reverse transcription using the ReadyScript™ cDNA synthesis mix (Sigma). The following forward and the reverse primers were obtained from Sigma Aldrich: mCyp1a2, forward (5'-CAATGACATCTTTGGAGCTG-3') and reverse (5'-GATGAAGGCCTCTAGATATGG-3'); mCyp2c29, forward (5'-CAGATGTCACAGCTAAAGTC-3') and reverse (5'-TTTAATGTCACAGGTCAGT-3'); mCyp2d22, forward (5'-GTTGTACTAAATGGGCTGAC-3') and reverse (5'-GCTAGGACTATACCTTGAGAG-3'); mCyp3a11, forward (5'-CTGACACCAGTATATGAGATG-3') and reverse (5'-GGCTTTATGAGAGACTTTGTC-3'); mActin-beta, forward (5'-ACGGCCAACCGTGAAAAGAT-3') and reverse (5'-GTGGTACGACCAGAGGCATAC-3'). qRT-PCR was performed on the LightCycler 480 system (Roche) as follows: one cycle of initial denaturation (4 min at 95°C), 45 cycles of amplification (10 sec at 95°C and 30 sec at 60°C), and a cooling period. The data presented are relative mRNA levels normalized to the level of Actin-beta, and the average value from the WT control group was set as 1.

Immunoblot protein preparation and analysis:

300 mg liver tissue was homogenized in NP40 lysis buffer (20 mM Tris-HCl, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, and 2 mM EDTA with 1 Protease Inhibitor Cocktail Tablet (Roche, Indianapolis, IN) per 25 ml) on the ice. Homogenized tissue was then centrifuged at 10,000g for 30 minutes, and then the supernatant was transferred to a new collection vial. Protein concentrations in the collected supernatant were quantified by the Pierce BCA Protein Quantitation Assay kit (Thermo Fisher Scientific, Waltham, MA) following the manufacturer's protocol.

50 µg of the whole cell lysate in each well were separated on 7.5% SDS-PAGE gels and transferred to polyvinylidene difluoride (PVDF) membranes. The following antibodies were used for the detection of proteins: Erk2 (Santa Cruz Biotechnology, Inc., Dallas, TX, sc-154), Cyp1a2 (sc-9835), Cyp3a (sc-25845), Cyp2d22 (Abcam, Cambridge, MA, Ab62204) and Cyp2c29 (Ab137015). Densitometry analysis of the western blot was performed using ImageJ software (National Institutes of Health, Bethesda, MD), and relative protein expression was calculated by normalizing to the housekeeping protein Erk2 (sc-154).

Concordance analysis across human and *ob/ob* MCD mice:

Concordance analysis was performed to compare diabetic NASH mice to human NASH on the enzymatic activity, mRNA expression and protein expression of the CYP isoforms: CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4. The analysis was conducted by measuring the effect size of NASH versus control, in the mice is the *ob/ob* MCD versus WT while in human is NASH with/without fatty versus normal, of either enzymatic activity, mRNA expression or protein expression. The effect size (estimated by Glass's D) is defined as the standardized mean difference between two populations and can be calculated using the following equation:

$$\Delta = \frac{\mu_1 - \mu_2}{S_p}$$

where μ_1 and μ_2 are the sample means for two groups, and S_p is the pooled standard deviation for both. Data of this study were compared to the published data in human (17). The analysis was performed using R version 3.2.3 with packages of gdata_2.17.0, xtable_1.8-0, ggplot2_1.0.1 and knitr_1.11 (<http://www.r-project.org/>).

Statistical analysis:

All results are represented as mean $\pm$ standard error of the mean (SEM). For all comparisons within this study One-way analysis of variance (ANOVA) statistical analysis was employed with a Tukey's multiple comparisons post-test to compare wild type versus *ob/ob* mice, wild type versus *ob/ob* MCD mice and *ob/ob* versus *ob/ob* MCD mice. All analysis was carried out using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA).

Results

1. NASH phenotype in mouse models

Hematoxylin and eosin stained liver tissue sections from representative WT, *ob/ob* and *ob/ob* MCD mice are shown in Figure 1A. Livers were evaluated and incidence/severity scored by a board certified veterinary pathologist (Figure 1B). Macrovesicular steatosis, a distinguishing characteristic of NASH, was only observed in the *ob/ob* MCD mice. The *ob/ob* MCD exhibited greater incidence/severity scores on each of the typical NASH histological diagnoses, including hepatic steatosis, inflammation, fibrosis, biliary hyperplasia and hepatocyte necrosis compared to the WT (Figure 1B). To determine the diabetic phenotype, plasma glucose and insulin levels were examined in each group of mice. *ob/ob* mice displayed a diabetic phenotype with higher levels insulin. The glucose levels significantly decreased in *ob/ob* MCD mice, but a significant change of insulin level was not observed in *ob/ob* MCD mice (Figure 1C).

2. *In vivo* Pharmacokinetics of a Drug Cocktail in NASH models

A single dose oral pharmacokinetics study was performed using a cocktail of CYP probe drugs to determine the activity of the respective CYP isoforms. Plasma concentration of the parent drugs (Caffeine, Losartan, Omeprazole, Midazolam and Dextromethophan) and their metabolites (Paraxanthine, EXP3174, 5-hydroxyomeprazole, 1-hydroxymidazolam and Dextrorphan) was measured by LC-MS/MS. The pharmacokinetic curves of each probe drug and metabolite are shown in Figure 2A and Figure 2B, respectively, and the pharmacokinetic parameters are summarized in Table 1. The maximum concentration (C_{max}) and the area under curve (AUC) of caffeine in diabetic and diabetic NASH mice were significantly increased, although the drug half-life ($t_{1/2}$) was not significantly changed in diabetic or diabetic NASH mice (Table 1). The

metabolism AUC ratio, defined as the ratio of metabolite AUC to parent drug AUC, of each probe drug was calculated to represent the respective enzymatic activity. The metabolism AUC ratio of caffeine and omeprazole were significantly decreased in diabetic and diabetic NASH mice compared to wild type mice. The metabolism AUC ratio of losartan was significantly decreased in the diabetic NASH mice compared to diabetic mice (Figure 2C).

3. Hepatic CYP mRNA expression alteration in diabetic NASH models

The hepatic mRNA expression of the major CYP isoforms, Cyp1a2, Cyp2c29, Cyp2d22 and Cyp3a11 were normalized to mRNA expression of beta-Actin, and the fold changes were shown in Figure 3. The Cyp1a2 mRNA expression level was significantly decreased in diabetic and diabetic NASH mice. The diabetic mice had a decreased mRNA level of Cyp2c29 compared to the wild type mice. Additionally, the mRNA level of Cyp2c29 was significantly decreased in the diabetic NASH mice compared to both the wild type and the diabetic mice. The mRNA level of Cyp3a11 was significantly increased in the diabetic mice compared to wild type.

4. Hepatic CYP protein content alteration in diabetic NASH models

Western blot analysis was performed to determine the relative protein content of the major CYPs in mice of each group (Figure 4A). The fold change of protein content of each CYP isoform, which is normalized to Erk protein content, was shown in Figure 4B. In the diabetic mice, the protein expression of Cyp1a2, Cyp2c29 and Cyp2d22 were significantly decreased. Furthermore, a significant decrease in protein content of Cyp1a2, Cyp2c29, Cyp2d22 and Cyp3a was observed in the diabetic NASH mice compared to the wild type mice. However, the only significant change in the diabetic NASH mice compared to the diabetic mice was the decreased Cyp2d22 protein content.

5. Concordance analysis across human and mouse CYP activities, mRNA and protein expression in NASH

To determine the extent to which the diabetic NASH model recapitulates the alterations in CYP mRNA, protein expression and enzymatic activities in human NASH, a concordance analysis was performed using previously published human NASH data(17). Since Cyp2c29 was reported as the mouse ortholog of both CYP2C9 and CYP2C19 in human(29, 30), Cyp2c29 was compared to both CYP2C9 and CYP2C19. Effect sizes in the same direction (the lower left or upper right quadrant) represent similar trends of change, whereas the magnitude of change corresponds to the statistical power in the detection of expression difference in NASH versus control. Figure 5A represents the comparison of enzymatic activities. Three pairs of CYP isoforms changed in the same direction, which are CYP1A2, CYP2C19 and CYP3A4 in human. The mRNA expression of Cyp1a2, Cyp2c29 and Cyp2d22 changed in the same direction as their corresponding human genes CYP1A2, CYP2C19 and CYP2D6, though the magnitude of that change varied widely (Figure 5B). While all the five comparisons pairs indicated the same direction of decreased protein expression (Figure 5C), there was large variability in the magnitude of that change.

Discussion

An ideal animal model that can accurately reflect the diverse population of patients with NASH is lacking, which limits pre-clinical risk assessment or predictability of VDRs in NASH patients. In this present study, we utilized the *ob/ob* MCD mouse model as our investigative tool, because it may predict both histomorphological and some of the metabolic or biochemical features of human NASH. The typical lesion of macro-vesicular steatosis as well as a certain degree of inflammation and fibrosis were repeated in the *ob/ob* MCD mice in this study as previously demonstrated (15, 33) (Figure 1A & 1B). Additionally, insulin and glucose levels were also consistent with the development of diabetes as previously shown (15) (Figure 1C).

Significant changes in probe drug pharmacokinetics were found in the diabetic NASH mice, suggesting alterations in the *in vivo* activities of their corresponding CYP isoforms. The pharmacokinetics of caffeine, the probe drug of Cyp1a2, as well as omeprazole and losartan, the two probe drugs of the Cyp2c subfamily, were significantly changed, directly indicating a decrease in enzymatic activity (Figure 2C). The mRNA expression of Cyp1a2 and Cyp2c29 were significantly decreased in both the obese diabetic mice and the diabetic NASH mice (Figure 3), while an overall decrease of protein expression of Cyp1a2, Cyp2c29, Cyp2d22 and Cyp3a11 was found in the diabetic NASH mice (Figure 4). Accordingly, data of enzymatic activity, mRNA expression and protein expression of the murine CYP isoforms are not in complete concordance with each other, which may be indicative of the heterogeneity in the regulation mechanisms known for each isoform. Others have reported altered CYP activities and expression levels in multiple rodent models of diabetes and NASH. A study by Watson *et al.* reported no significant change of Cyp1a activity in *ob/ob* mice (34). A separate study in MCD mice reported a decrease in mRNA expression of Cyp1a2 and an increase in Cyp3a11 (35). Lam *et al* reported a

significant increase in Cyp2c29 mRNA expression in 10-week old *db/db* mice, which are the leptin receptor deficient mice exhibiting similar diabetic phenotype as *ob/ob* mice, and a significant decrease in expression in 25-week old *db/db* mice compared to their 25-week old control (36). Yoshinari observed an increase in both mRNA and protein expression of Cyp2c29 in 10-week old *db/db* mice (37). Lastly, a microarray study, intended to identify the general gene expression alterations in the *ob/ob*, *db/db* and HFD-fed C57BL/6J mice, revealed that the dramatically changed genes were highly interconnected and significantly enriched for processes involving metabolism by CYPs (38). Collectively, these data indicate that, while there are similarities between mouse models of diabetes and NASH, they are not all the same and care should be taken to understand the changes in CYP-mediated drug metabolism.

The concordance analysis between mouse *in vivo* and previously published human *ex vivo* data for CYP activity shows that even though the diabetic NASH mouse model we used in this study has been demonstrated to accurately reflect the drug transporter alteration profiles in humans, it partially recapitulates the alteration pattern of CYP1A2, CYP2C19 and CYP3A4, but fails to resemble the alterations of CYP2C9 and CYP2D6 observed in human NASH.

In conclusion, while the *ob/ob* MCD model accurately recapitulates certain aspects of human NASH including insulin insensitivity, pathological hallmarks, and transporter expression profiles, it cannot precisely reflect the alteration in the major CYP activities for drug metabolism. Studies modeling the effect of NASH on the disposition of drugs through the action of drug transporters may be highly relevant to human NASH patients, but caution should be used when extrapolating any CYP-mediated drug metabolism. More comprehensive human studies should be performed to evaluate the *in vivo* CYP activity in NASH patients. Additional effort should be devoted to

establish a more accurate model of CYP regulation in the progression of NASH to ensure the safety of this at risk patient population.

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Figure Legends:

Figure 1: Histological and pathological features of NASH in WT, *ob/ob* and *ob/ob* MCD mice. Pictures of representative hematoxylin and eosin stained liver sections from WT, *ob/ob* and *ob/ob* MCD mice were taken in original magnification of 20X(A). Histopathological analysis of liver from WT, *ob/ob* and *ob/ob* MCD mice on steatosis, necrosis, inflammation, fibrosis and biliary hyperplasia (B). Plasma collected from WT, *ob/ob* and *ob/ob* MCD mice was analyzed for peripheral blood glucose and insulin concentrations (C). Data represent mean $\pm$ SEM. WT n=5, *ob/ob* n=5, *ob/ob* MCD n=5. One-way ANOVA with Tukey's multiple comparisons post-test were performed to determine the statistical significance. * Indicates a significant difference between *ob/ob* and/or *ob/ob* MCD with WT mice, while # indicates a significant difference between *ob/ob* and *ob/ob* MCD mice.

Figure 2: Pharmacokinetics of the five probe drugs and their metabolites. Pharmacokinetics curves of the five probe drugs: caffeine, omeprazole, midazolam, losartan and dextromethorphan (A) and their metabolites: paraxanthine, 5-hydroxy-omeprazole, 1-hydroxy-midazolam, EXP-3174 and dextrophan (B) were graphed based on the time-course plasma concentrations. The AUC ratio of each pair of probe drug and the metabolite, calculated based on the AUC of each pharmacokinetic curve of each compound, represent the relative enzymatic activity of the corresponding CYP isoform (C). Data represent mean $\pm$ SEM. WT n=5, *ob/ob* n=5, *ob/ob* MCD n=5. One-way ANOVA with Tukey's multiple comparisons post-test were performed to determine the statistical significance. * Indicates a significant difference between *ob/ob* and/or *ob/ob* MCD with WT mice, while # indicates a significant difference between *ob/ob* and *ob/ob* MCD mice.

Figure 3: Hepatic mRNA expression of murine CYP isoforms in WT, *ob/ob* and *ob/ob* MCD mice. Hepatic mRNA expression of Cyp1a2, Cyp2c29, Cyp2d22 and Cyp3a11 was quantified and normalized to beta-Actin in WT, *ob/ob* and *ob/ob* MCD mice. Data represent mean $\pm$ SEM. WT n=5, *ob/ob* n=5, *ob/ob* MCD n=5. One-way ANOVA with Tukey's multiple comparisons post-test were performed to determine the statistical significance. * Indicates a significant difference between *ob/ob* and/or *ob/ob* MCD with WT mice, while # indicates a significant difference between *ob/ob* and *ob/ob* MCD mice.

Figure 4: Hepatic protein content of murine CYP isoforms in WT, *ob/ob* and *ob/ob* MCD mice. Hepatic protein expression of Cyp1a2, Cyp2c29, Cyp2d22 and Cyp3a11 were measured in WT, *ob/ob* and *ob/ob* MCD mice by western blot (A). Densitometry analysis of western blot was performed, and relative protein expression of each CYP isoform to Erk protein was shown (B). Data represent mean $\pm$ SEM. WT n=5, *ob/ob* n=5, *ob/ob* MCD n=5. One-way ANOVA with Tukey's multiple comparisons post-test were performed to determine the statistical significance. * Indicates a significant difference between *ob/ob* and/or *ob/ob* MCD with WT mice, while # indicates a significant difference between *ob/ob* and *ob/ob* MCD mice.

Figure 5: Effective size analysis of *ob/ob* MCD mouse and human enzymatic activity, mRNA expression and protein expression of five CYP isoforms. Comparison of effect size of human NASH on CYP isoform enzymatic activity (A), mRNA expression (B) and protein expression (C) changes versus the diabetic NASH mouse model changes. Positive effect reflects increase, whereas negative effect changes reflect decrease. The points in the lower left and upper right quadrant represent the same direction of changes, while the dots in the upper left or lower right quadrant represent the opposite direction of changes. Values were acquired by the method described in the materials and method section.

Table 1: Comparison of pharmacokinetics parameters of the probe drugs.

Non-compartmental analysis was applied to calculate area under curve (AUC), terminal half-life ($t_{1/2}$), maximum plasma concentration ($C_{\max}$), and the AUC ratio was calculated based on the AUC value of the metabolite and the parent drug.

	Group	Cmax ($\mu\text{g/mL}$)	AUC($\text{min}\cdot\mu\text{g/mL}$)	AUCmetabolite/ AUCdrug	T1/2 (min)
	WT	2.0 $\pm$ 1.1	491.1 $\pm$ 91.3	1.06 $\pm$ 0.03	158 $\pm$ 71
Caffeine	<i>ob/ob</i>	4.9 $\pm$ 0.9 *	1418.0 $\pm$ 341.8 *	0.46 $\pm$ 0.17 *	342 $\pm$ 236
	<i>ob/ob</i> MCD	5.1 $\pm$ 1.2 *	1431.0 $\pm$ 325.7 *	0.67 $\pm$ 0.34 *	221 $\pm$ 131
	WT	0.6 $\pm$ 0.7	36.2 $\pm$ 31.7	1.28 $\pm$ 0.76	70 $\pm$ 45
Omeprazole	<i>ob/ob</i>	3.1 $\pm$ 1.9	314.5 $\pm$ 195.4	0.36 $\pm$ 0.18 *	56 $\pm$ 13
	<i>ob/ob</i> MCD	4.7 $\pm$ 4.8	486.8 $\pm$ 450.7	0.52 $\pm$ 0.34 *	77 $\pm$ 31
	WT	0.30 $\pm$ 0.15	36.7 $\pm$ 11.8	6.70 $\pm$ 0.69	88 $\pm$ 38
Midazolam	<i>ob/ob</i>	0.26 $\pm$ 0.10	47.9 $\pm$ 18.5	7.30 $\pm$ 2.60	140 $\pm$ 53
	<i>ob/ob</i> MCD	0.47 $\pm$ 0.27	66.1 $\pm$ 37.2	5.71 $\pm$ 3.33	113 $\pm$ 32
	WT	0.15 $\pm$ 0.13	64.6 $\pm$ 115.1	1.38 $\pm$ 1.00	114 $\pm$ 51
Losartan	<i>ob/ob</i>	0.94 $\pm$ 1.12	123.9 $\pm$ 109.0	3.48 $\pm$ 1.97	90 $\pm$ 32
	<i>ob/ob</i> MCD	7.8 $\pm$ 10.50	1006.0 $\pm$ 1135.0	0.68 $\pm$ 0.41 [#]	132 $\pm$ 69
	WT	0.08 $\pm$ 0.03	19.1 $\pm$ 7.8	0.59 $\pm$ 0.14	123 $\pm$ 30
Dextromethorphan	<i>ob/ob</i>	0.17 $\pm$ 0.10	37.0 $\pm$ 23.3	0.56 $\pm$ 0.19	120 $\pm$ 35
	<i>ob/ob</i> MCD	0.36 $\pm$ 0.43	65.9 $\pm$ 55.3	0.71 $\pm$ 0.28	172 $\pm$ 131

Data represent mean $\pm$ SEM. WT n=5, *ob/ob* n=5, *ob/ob* MCD n=5. One-way ANOVA with Tukey's multiple comparisons post-test were performed to determine the statistical significance.

* Indicates a significant difference between *ob/ob* and/or *ob/ob* MCD with WT mice, while # indicates a significant difference between *ob/ob* and *ob/ob* MCD mice.

Figure 1.

A

B

C

Figure 2.

A

B

C

1-way ANOVA	Caffeine Paraxanthine	Losartan Exp3174	Omeprazole 5-hydroxyomeprazole	Dextromethorphan Dextrophan	Midazolam 1-hydroxymidazolam
P value	0.0653	0.0136	0.0263	0.4953	0.3880

Figure 3

1-way ANOVA	Cyp1a2	Cyp2c29	Cyp2d22	Cyp3a11
P value	<0.0001	0.0002	0.1402	0.0522

Figure 4

A

B

1-way ANOVA	Cyp1a2	Cyp2c29	Cyp2d22	Cyp3a
P value	<0.0001	<0.0001	0.0023	0.0037

Figure 5