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Ablation of both Oct1 and Oct2 alters metformin pharmacokinetics but has no effect on tissue drug exposure and pharmacodynamics

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Running title: Metformin PK/PD following ablation of both Oct1 and Oct2 (57/60)

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Abbreviations:

OCT, organic cation transporter; TEA, tetraethylammonium; GFR, glomerular filtration rate

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Abstract

OCT1 and OCT2 mediate hepatic uptake and secretory renal clearance of metformin, respectively. PK/PD implications of simultaneous impairment of both transporters, such as by systemic pan-OCT inhibition, have not been studied directly. At present metformin PK/PD, distribution, and excretion were studied in Oct1/Oct2-knockout mice. Metformin clearance was reduced 4.5 fold from renal blood flow to glomerular filtration rate (GFR), and volume of distribution was reduced 3.5 fold in Oct1/Oct2-knockout mice. Oral bioavailability was not affected (F = 64 ± 4 vs. 59 ± 11; knockout vs. wild type). Liver- and kidney-to-plasma concentration ratios were decreased in Oct1/Oct2-knockout mice 4.2 and 2.5 fold, respectively. Surprisingly, 2.9-fold increase in oral metformin exposure and reduced tissue partitioning yielded little-to-no net change in tissue drug concentrations. Absolute kidney exposure was unchanged (knockout:wild type = 1.1 ± 0.2), and liver exposure was only modestly decreased (knockout:wild type = 0.6 ± 0.1). Oral glucose AUC lowering by metformin was not impaired in Oct1/Oct2-knockout mice at the five dose levels tested [ED_{50} = 151 vs. 110 mg/kg; glucose lowering at highest dose = 42 ± 1 vs. 39 ± 4%; knockout vs. wild type); however, higher systemic metformin exposures were necessary in knockout mice to elicit the same effect ($EAUC_{50}$ = 70 vs. 26 μg*hr/mL). Despite major changes in metformin clearance and volume of distribution in Oct1/Oct2-knockout mice, tissue drug exposure and pharmacodynamics were not affected. These findings challenge the presumption that systemic OCT inhibition will affect metformin pharmacology.

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Introduction

Organic cation transporters (OCTs) mediate the electrogenic uptake of hydrophilic type I organic cations (Grundemann et al., 1994; Sweet and Pritchard, 1999). Three OCT isoforms have been identified, and the clinical importance of OCT1 and OCT2 in pharmacokinetics and/or drug interactions has been documented (Somogyi et al., 1987; Shu et al., 2007; 2008; Chen et al., 2009). OCT1 is present on the sinusoidal (basolateral) membrane of hepatocytes and is responsible for hepatic uptake of substrates (Jonker et al., 2001; Nishimura and Naito, 2005). Human OCT2 is expressed primarily on the basolateral membrane of renal tubules; in rodents in addition to Oct2, Oct1 is co-expressed in the kidney (Grundemann et al., 1994; Jonker et al., 2003). Considerable substrate and inhibitor overlap exists between OCT1 and OCT2. For example, prototypic substrates metformin and 1-methyl-4-phenylpyridinium, commonly known as MPP+, are transported by both OCT1 and OCT2 (Konig et al., 2011); cimetidine, amantadine, quinidine, and procainamide are equipotent inhibitors of both OCT isoforms (Minematsu et al., 2010).

Murine knockout models have been developed to interrogate the PK/PD relevance of OCT transport (DeGorter and Kim, 2011). Studies with Oct1-knockout mice demonstrated the importance of this isoform in hepatic distribution of several prototypic substrates, including tetraethylammonium (TEA), meta-iodobenzylguanide, 1-methyl-4-phenylpyridinium, and metformin (Jonker et al., 2001; Wang et al., 2002). However, no apparent impairment in the renal clearance of these compounds was observed in Oct1-knockout mice, presumably due to compensation by Oct2 (Jonker et al., 2001; Wang et al., 2002). Pronounced pharmacokinetic consequences of genetic ablation of Oct2 in mice were not observed (Jonker et al., 2003). As expected, hepatic distribution of TEA was not affected in Oct2-knockout mice, but surprisingly

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neither was renal clearance, because apical urinary secretion via multidrug and toxin extrusion protein appears to determine the overall rate of TEA tubular secretion even when only Oct1 uptake is maintained (Schali et al., 1983; Jonker et al., 2003; Tanihara et al., 2007). Thus to better understand the importance of Octs, the Oct1/Oct2 double-knockout mouse was developed (Jonker et al., 2003). Systemic exposure to TEA was increased significantly in Oct1/2-knockout mice and clearance was decreased from renal blood flow in wild-type mice to unbound GFR in the double-knockouts, consistent with elimination of the secretory component of renal clearance (Jonker et al., 2003).

Metformin is a commonly-used antidiabetic drug, whose pharmacokinetics are influenced by OCT transport (Shu et al., 2007; 2008; Chen et al., 2009). Initial studies in Oct1-knockout mice indicated that metformin hepatic exposure may be reduced as much as 30 fold (Wang et al., 2002), although subsequent reports estimated a less pronounced decrease of 2.5-8 fold in liver partitioning (Wang et al., 2003; Shu et al., 2007). Since hepatic distribution of metformin is necessary for its inhibition of gluconeogenesis, Oct1-knockout mice exhibited markedly attenuated metformin pharmacodynamics, including metformin-insensitive hepatocyte gluconeogenesis, abolished fasting glucose lowering effects, and reduced lactic acidosis (Wang et al., 2003; Shu et al., 2007). Systemic pharmacokinetics and renal clearance of metformin were not altered in Oct1-knockout mice (Wang et al., 2002; 2003; Shu et al., 2007), although significantly higher initial concentration following IV bolus administration suggested a decreased volume of distribution (Wang et al., 2002). Clinically, the oral volume of distribution of metformin was reduced 2.2 fold in subjects with functional OCT1 variants (Shu et al., 2007; 2008). Although metformin PK and/or PD studies in Oct2-knockout models are lacking, because humans do not express OCT1 along with OCT2 in the kidney, secretory renal clearance of

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metformin can be reduced up to 2.6 fold in people with functional OCT2 variants (Song et al., 2008; Chen et al., 2009).

Previous literature reports established the isolated importance of OCT1 in metformin hepatic distribution/pharmacodynamics and OCT2 in its secretory renal clearance. However, the effect of combined OCT1 and OCT2 impairment on metformin PK/PD and tissue distribution has not been studied systematically, even though non-specific OCT inhibitors will impair both isoforms. For example cimetidine, an equipotent inhibitor of OCT1 and OCT2 (Minematsu et al., 2010), increased metformin exposure 50% due to inhibition of secretory renal clearance (Somogyi et al., 1987; Wang et al., 2008), but lactic acidosis was not attenuated overall, and was actually significantly enhanced at one of four time points (Somogyi et al., 1987). These findings are surprising because clinically-notable inhibition of metformin renal clearance by cimetidine would also be expected to reduce hepatic distribution, and in turn, impair pharmacodynamics. In order to understand PK/PD alterations during combined impairment of both OCT1 and OCT2, metformin pharmacokinetics/dynamics were for the first time studied in Oct1/Oct2 double-knockout mice.

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Materials and Methods

Chemicals: Tetraethylammonium (TEA) and dextrose were purchased from Sigma-Aldrich (St. Louis, MO). Metformin-HCl and D_6-metformin-HCl were purchased from Toronto Research Chemicals (North York, Ontario). [^{14}C]inulin was obtained from PerkinElmer (Waltham, MA). All other chemicals were of reagent grade and were readily available from commercial sources.

Animals: Age-matched Oct1/Oct2 double-knockout and wild-type FVB male mice were purchased from Taconic (Hudson, NY). Mice were delivered to Covance (Greenfield, IN), where they were acclimated for at least 72 hours prior to initiation of PK studies or 2 weeks prior to PK/PD studies in order to ensure the same normal diet. In all cases, mice were between 6-8 weeks of age at time of study. All animal procedures were approved by the Institutional Animal Care and Use Committee at Covance.

IV Pharmacokinetics and Excretion: TEA was dissolved in phosphate-buffered saline (pH = 7.4) and was administered to mice as a tail vein injection (10 mg/kg; 10 mL/kg). Blood samples were collected at the following times: 3, 6, 10, 15, 20, 30, 40, 50, and 60 minutes post dose administration. Metformin was dissolved in phosphate-buffered saline (pH = 7.4) and was administered to mice as a tail vein injection (5 mg/kg; 10 mL/kg). Blood samples were collected at the following times: 3, 6, 10, 20, 30, 45, 60, 90, and 120 minutes post dose administration. In all IV pharmacokinetic studies, three time points were collected per mouse, the first two were retro-orbital bleeds and the third blood draw was performed by cardiac puncture. As such, the plasma concentration-time data are a composite of three groups of four mice (n = 4 mice/group/time point).

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In urinary excretion studies, mice received a bolus intravenous dose of TEA (10 mg/kg) or metformin (5 mg/kg) as described above. Following dose administration, mice were housed in metabolic cages (n = 4 metabolic cages each containing 3 mice; 12 mice total per group). Urine was collected *in toto* from 0-2 hours post dose administration, when a bladder stick was conducted to remove residual urine from the bladder, and metabolic cages were washed with 50% methanol. Endogenous creatinine renal clearance, as well as [^{14}C]inulin renal clearance following a 100 μCi intravenous bolus dose, were determined by measuring total urinary output over a 24-hour period along with the corresponding systemic exposure (serum creatinine or plasma radioactivity) over the urine collection interval. Urine and serum creatinine was quantified using the Cobas 6000 system (Roche Diagnostics, Indianapolis, IN); [^{14}C]inulin levels were measured by scintillation counting.

Metformin PK/PD and Tissue Distribution: Metformin oral PK/PD and tissue distribution studies were conducted on three separate days with one-week recovery period in between. The study design outlined below was repeated for each metformin oral dose level (10, 30, 60, 100, 300 mg/kg). Each metformin dose level consisted of a total of 12 Oct1/2-knockout and 12 wild-type mice. In order to ensure the same normal diet between Oct1/2-knockout and wild-type mice, animals were ordered between 4-6 weeks of age and were acclimated for 2 weeks prior to the beginning of these studies. On each study day, mice were fasted for 16 hours prior to study initiation, and throughout the duration of the 2.5-hour study.

On study Day 1, control glucose and insulin AUC were determined following on oral glucose tolerance test in the absence of metformin. At -30 minutes, mice received metformin vehicle by oral gavage (1% hydroxyethyl cellulose/0.25% polysorabte-80/0.05% antifoam in water; 10 mL/kg). At 0 minutes, the oral glucose tolerance test was initiated by oral gavage

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(25% dextrose in water; 2.5 g/kg; 10 ml/kg). Blood glucose and plasma insulin levels were measured at 0, 30, 60, 90, and 120 minutes post glucose tolerance test. Blood glucose concentrations were measured with a strip glucometer. Plasma insulin levels were measured using the mouse/rat insulin immunoassay kit (Meso Scale Diagnostics; Gaithersburg, MD).

On study Day 8, glucose and insulin AUC were determined following on oral glucose tolerance test with metformin pre-treatment. The study design was identical to Day 1, except at -30 minutes, mice received metformin by oral gavage (10, 30, 60, 100, or 300 mg/kg; 10 mL/kg).

On study Day 15, metformin pharmacokinetics, as well as liver and kidney exposures were determined following administration of metformin by oral gavage (10, 30, 60, 100, or 300 mg/kg; 10 mL/kg). Glucose tolerance test was not administered on study Day 15. Blood samples were collected at the following time points: 5, 10, 20, 30, 45, 60, 90, 120, and 150 minutes post metformin administration. In all pharmacokinetic studies, three time points were collected per mouse, the first two were retro-orbital bleeds and the third blood draw was performed by cardiac puncture. As such, the plasma concentration-time profiles are a composite of three groups of four mice (n = 4 mice/group/time point). At the time of terminal blood collection (90, 120, and 150 min), liver and kidneys were excised for analysis of tissue metformin levels. Likewise, the tissue concentration-time profiles are a composite of three groups of four mice (n = 4 mice/group/time point).

Bioanalysis: TEA and metformin in plasma, urine, cage wash, liver and kidney tissue homogenate was analyzed by LC-MS/MS. All samples were mixed with an organic internal standard solution to precipitate protein, centrifuged, and the resulting supernatants were directly analyzed. TEA and its internal standard, N,N-trimethyl-phenylalaninamide, were eluted from a C18 column [Aquasil 2.1x20 mm, 5 μm (Thermo Fisher Scientific, Inc.; Waltham, MA)] with a

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mobile phase gradient. Metformin and its internal standard, D_6-metformin, were eluted from a C18 column [Betasil Si 2.1x50 mm, 5 μm (Thermo Fisher Scientific, Inc.; Waltham, MA)] with a mobile phase gradient optimized for each matrix. Analytes were detected in positive ion mode using multiple reaction monitoring [Sciex API 4000 triple quadrupole mass spectrometer equipped with a TurboIonSpray interface (Applied Biosystems/MDS; Foster City, CA)]: TEA 130.1 → 86.1 m/z and N,N-trimethyl-phenylalaninamide 207.1 → 148.1 m/z; metformin 130.1 → 71.1 and D_6-metformin 136.1 → 77.1 m/z. The dynamic range of the assays was 1-5000 ng/mL for both TEA and metformin in all relevant matrices. Samples with analyte concentrations above the upper limit of quantification were diluted with matrix to within the assay range; concentrations below the lower limit of quantification were reported as such.

Data Analysis: Noncompartmental composite pharmacokinetic parameters were calculated using Watson v. 7.4 (Thermo Scientific; Waltham, MA). All renal clearances were calculated as the ratio of total amount excreted in urine and systemic exposure measured over the corresponding urine collection time interval. The simple Emax model was fitted to the pharmacodynamic data using WinNonlin v. 5.3 (Pharsight; Cary, NC). Since pharmacokinetic parameters were calculated from mean values in composite concentration-time datasets (see above), only mean parameter estimates are reported without variability estimates and significance testing. Otherwise all data are reported as mean ± S.E.M., with the corresponding n noted. S.E.M. was selected as the variability descriptor, because many reported parameters represent the mean of means (ex. oral bioavailability).

Significance testing was carried out in SigmaPlot v. 11.0 (Systat Software Inc.; Chicago, IL). The Student's two-tailed t-test was used to assess statistical significance between two groups. In cases where variance was different between compared groups (F-test, $p < 0.05$), the

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unequal-variance t-test was used. Significance of changes in composite tissue exposures, knockout relative to wild type, was assessed with a one-sample t-test. Significance of pharmacodynamic data, which were affected by two factors [mouse type (knockout or wild type) and drug treatment (metformin or vehicle)], was assessed with a 2-way analysis of variance with Tukey's post hoc test. In all cases, the minimal criterion for significance was $p < 0.05$.

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Results

TEA concentration-time profiles in wild-type and Oct1/2-knockout mice following administration of a 10-mg/kg TEA IV bolus dose are presented in Figure 1; corresponding PK parameters are summarized in Table 1. Knockout mice exhibited significantly higher TEA systemic concentrations at all nine time points; overall systemic exposure was 3.8 fold higher. TEA systemic clearance in wild-type mice was rapid and comparable to renal blood flow, but was reduced 3.5 fold in Oct1/2-knockout mice to approximately unbound GFR (Davies and Morris, 1993; Jonker et al., 2003). Volume of distribution was 5 fold lower in Oct1/2-knockout mice. Due to both decreased clearance and volume, the terminal half-life was not markedly different in knockout mice but was nonetheless reduced 30% reflecting the greater magnitude of decrease in volume than clearance. TEA urinary excretion over 2 hours was only 16% lower in knockout mice; considering the 3.8 fold higher systemic exposure, renal clearance was reduced from 5 times GFR in wild-type mice to approximately GFR. With the functional absence of Oct1 and Oct2 in the commercially-obtained Oct1/2-knockout mice thus confirmed (Jonker et al., 2003), metformin studies were commenced.

Metformin intravenous pharmacokinetics were studied in wild-type and Oct1/2-knockout mice following administration of a 5-mg/kg IV bolus dose (Figure 2A and Table 2). Knockout mice exhibited significantly higher metformin systemic concentrations at all nine time points; systemic exposure was on average 4.4 fold higher. Systemic clearance in wild-type mice was slightly higher than renal blood flow, but was reduced 4.5 fold in Oct1/2-knockout mice to approximately unbound GFR (Davies and Morris, 1993; Glucophage (metformin hydrochloride) prescribing information, 2009). Volume of distribution was 3.5 fold lower in knockout mice. Terminal half-life was comparable between wild-type and Oct1/2-knockout mice, which is

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consistent with a similar magnitude of decrease in both clearance and volume. Over 2 hours post intravenous dose administration, metformin urinary excretion was only 24% lower in Oct1/2-knockout mice; however, due to 4.5 fold higher systemic exposure, renal clearance was reduced from 4 times GFR in wild-type mice to approximately GFR.

Metformin oral pharmacokinetics were studied in wild-type and Oct1/2-knockout mice at five dose levels (Figure 2B-D and Table 2). Metformin concentration-time curve following administration of a 10-mg/kg oral dose (Figure 2B) was strikingly different in appearance from the intravenous profile (5 mg/kg IV, comparable exposure to 10 mg/kg PO; Figure 2A) and is characterized by apparent protracted absorption. Concentrations at 5- and 10-minutes post oral dose were not significantly increased, but all plasma concentrations at subsequent time points were higher in Oct1/2-knockout mice ($p < 0.05$). Oral exposure increased in a dose-proportional manner between 10 and 300 mg/kg (Figure 2C) and was on average 2.9 ± 0.2 fold higher in Oct1/2-knockout mice ($p < 0.05$). Likewise, Cmax increased in a dose-proportional manner (Figure 2D) and was 2.4 ± 0.2 fold higher in Oct1/2-knockout mice ($p < 0.05$). Oral bioavailability and Tmax values showed no apparent dose-dependence in the 10-300-mg/kg range tested and were comparable between wild-type and Oct1/2-knockout mice (Table 2).

Metformin liver and kidney tissue-to-plasma concentration ratios were markedly decreased in Oct1/2-knockout mice (Figures 3-4). Significant differences were noted in the tissue-to-plasma concentration ratios with respect to time and dose level, and so these values are presented individually. Liver metformin partitioning was significantly reduced in Oct1/2-knockout mice in 13 out of 15 measurements; overall liver-to-plasma ratio was significantly decreased with a median 4.2-fold reduction (Figure 3). At all time points, a consistent trend of decreased liver metformin partitioning was noted in both wild-type and Oct1/2-knockout mice at

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the highest 300-mg/kg dose relative to the four lower dose levels, suggesting saturation of hepatic uptake. The magnitude of decrease in kidney partitioning was less pronounced. Kidney-to-plasma metformin concentration ratio was significantly reduced in Oct1/2-knockout mice in 7 out of 15 measurements; overall this ratio was significantly decreased with a median decrease of 2.5 fold (Figure 4). A trend of decreased kidney metformin partitioning was noted in both wild-type and Oct1/2-knockout mice at the highest 300-mg/kg dose, suggesting saturation of renal uptake. Note that in Figures 3-4 unusually high tissue concentrations were observed in some animals at the 100-mg/kg dose level, so the mean values are skewed upwards and the associated variability is high at this dose.

Surprisingly, absolute liver and kidney tissue metformin exposures were not markedly reduced in Oct1/2-knockout mice (Figure 5). Hepatic exposure was modestly lower in Oct1/2-knockout mice at each dose level tested (Figure 5A), but on average this decrease was only 42 ± 6%. Although the decrease itself was statistically significant, both absolute and dose-normalized liver metformin exposures were not significantly different between wild-type and Oct1/2-knockout mice. Kidney drug exposure was comparable at all five dose levels tested between wild-type and Oct1/2-knockout mice (Figure 5B); on average the knockout/wild-type exposure ratio was 1.1 ± 0.2.

The effects of metformin on blood glucose levels following an oral tolerance test in wild-type and Oct1/2-knockout mice are summarized in Figure 6. At the lowest 10-mg/kg dose, metformin did not significantly lower blood glucose concentrations (Figure 6A); however, at the highest 300-mg/kg dose, metformin significantly decreased blood glucose levels at all time points following the oral tolerance test and maintained them close to baseline (Figure 6B). Surprisingly glucose AUC lowering by metformin was not impaired in Oct1/2-knockout mice at

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any of the five dose levels tested (ED_{50} = 151 vs. 110 mg/kg, knockout vs. wild type), and the effect on blood glucose levels over the tested dose range was generally comparable (Figure 6C). Likewise, the observed metformin maximal effect was not attenuated in Oct1/2-knockout mice (glucose AUC lowering at highest 300-mg/kg dose = 42 ± 1 vs. 39 ± 4%, knockout vs. wild type; Figures 6B-C). Although the glucose lowering effect-dose relationship was unchanged between wild-type and Oct1/2-knockout mice, higher systemic metformin exposures were necessary to achieve the same effect in knockouts ($EAUC_{50}$ = 70 vs. 26 μg*hr/mL, knockout vs. wild type; Figures 6D).

To confirm that the observed metformin glucose-lowering effects (Figure 6) were not mediated by insulin modulation, plasma insulin concentrations were monitored. As expected, insulin levels following the glucose tolerance test were not markedly different between wild-type and Oct1/2-knockout mice (Figure 7). Although metformin significantly decreased insulin exposure in wild-type relative to Oct1/2-knockout mice at the highest 300-mg/kg dose level, no other significant differences or overall trends were otherwise noted.

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Discussion:

Previous literature reports established the importance of OCT1 in metformin hepatic distribution, necessary for its pharmacological activity, and OCT2 in its secretory renal clearance (Wang et al., 2002; 2003; 2008; Shu et al., 2007; 2008; Song et al., 2008); however, the PK/PD relevance of each OCT isoform was evaluated independently. These studies are sufficient to support the interpretation of OCT1 or OCT2 functional variants in the context of metformin distribution/pharmacology or renal clearance. However, many OCT inhibitors are nonspecific (Minematsu et al., 2010), and the effect of pan-OCT inhibition on metformin PK/PD and tissue distribution has not been thoroughly evaluated. To gain insight into this issue, at present metformin pharmacokinetics/dynamics were studied in a model with genetic ablation of both Oct1 and Oct2.

Oct1/Oct2-double knockout phenotype was confirmed with TEA pharmacokinetics and urinary excretion properties previously reported by Dr. Alfred H. Schinkel's laboratory, who initially generated the mice used in the current studies (Jonker et al., 2003). The present results are in agreement with the previous report and confirm the Oct1/Oct2 double-knockout phenotype, which with respect to TEA disposition is distinctly different from Oct1/Oct2 +/+, as well as Oct1 and Oct2 single-knockout phenotypes (Jonker et al., 2003). Considering the unexpected findings with respect to absolute metformin tissue exposure and pharmacodynamics, confirmation of the functional absence of both Oct genes was necessary.

Oct1/2-knockout mice demonstrated the expected alterations in metformin systemic pharmacokinetics, as well as liver and kidney distribution (Wang et al., 2002; 2003; 2008; Shu et al., 2007; 2008; Song et al., 2008). Knockout mice exhibited significantly higher metformin systemic concentrations due to a 4.5-fold decrease in systemic clearance to approximately the

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glomerular filtration rate, and a 3.5-fold decrease in the volume of distribution. These observations are consistent with up to 2.6-fold reduction in secretory renal clearance in humans with a functional OCT2 variant (Song et al., 2008; Chen et al., 2009), as well as 2.2-fold decrease in oral volume of distribution in people with a functional OCT1 variant (Shu et al., 2007; 2008).

In Oct1/2-knockout mice, hepatic and renal tissue partitioning of metformin was reduced 4.2 and 2.5 fold, respectively. Metformin liver-to-plasma concentration ratio has been suggested to be 30 fold decreased in Oct1-knockout mice (Wang et al., 2002), but this measurement was made during the distributional phase. Subsequent studies properly estimated the extent of attenuation in hepatic metformin distribution in Oct1-knockout mice to be 2.5, 4, and 8 fold (Wang et al., 2003; Shu et al., 2007), which is consistent with the 4.2 fold reduction in the present study. Direct reference for the 2.5-fold decrease in kidney-to-plasma concentration ratio in the Oct1/2-knockout mouse does not exist in the literature, but it is consistent with up to 2.6-fold reduction in secretory renal clearance in humans with a functional OCT2 variant (Song et al., 2008; Chen et al., 2009).

Based on the observed decreases in liver and kidney partitioning, and assuming no compensatory changes in other metformin hepatic and renal transport mechanisms in the Oct1/2-knockout mouse, the fraction excreted calculation (Zamek-Gliszczynski et al., 2009) can be used to estimate the contribution of Oct's to hepatic and renal uptake of metformin. Thus, Oct1 is estimated to mediate 76% of metformin hepatic uptake in mice, while Oct1 and Oct2 together account for 60% of metformin renal uptake. These estimates are consistent with the existing literature supporting these transporters as the major hepatic and renal uptake pathways for metformin (Giacomini et al., 2010). However, they also highlight the existence of additional

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non-Oct1/2 uptake mechanism(s), which may account for as much as 24% of hepatic and 40% of renal metformin uptake. Passive diffusion into hepatocytes and cells of the renal proximal tubule is highly unlikely; metformin is very polar (LogD = -6.13) and exhibited artificial membrane permeability that was too low to quantify ($< 0.27x10^{-6}$ cm/s) (Saitoh et al., 2004). Although OCT3 has not been reported to be an important determinant of drug pharmacokinetics (Giacomini et al., 2010), it is capable of transporting metformin *in vitro* and is expressed in the liver and kidney (Nishimura and Naito, 2005; Nies et al., 2009; Chen et al., 2010). Oct3 is the most logical mechanism behind the observed residual non-Oct1/2-uptake of metformin, although additional pathways cannot be ruled out.

A common concern with knockout animal models are the expression changes that may occur to compensate for the absent protein (Giacomini et al., 2010). In the case of metformin pharmacokinetics in the Oct1/2-knockout mice, potential changes to Mate's are most relevant (Kusuhara et al., 2011). In the present study both metformin renal tissue exposure and urinary recovery were essentially unchanged in Oct1/2-knockout mice. Since $dX_{urine}/dt = CL_{apical}xC_{kidney}$, the only way both renal tissue exposure (C_{kidney}) and urinary recovery (X_{urine}) can remain unchanged is if the excretory clearance for transport between tubular cells and urine (CL_{apical}) is not altered in the knockout mice. Since urinary recovery and kidney tissue concentration were similar in the knockouts, renal Mate function was not altered to a notable extent.

Metformin pharmacology is complex and multifaceted (Bailey and Turner, 1996). Three major mechanisms of action are involved: inhibition of hepatic gluconeogenesis, stimulation of peripheral glucose uptake and utilization, and inhibition of intestinal glucose absorption (Glucophage (metformin hydrochloride) prescribing information, 2009). These complexities

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raise the question whether mice are a representative species of metformin clinical PK/PD. Key metformin systemic PK parameters are in good agreement between mice and humans: clinical oral bioavailability was 50-60% vs. 59-64% in mice; metformin was well distributed in both species with a volume of distribution of 4.7 ± 2.6 L/kg in humans (oral V/F x F) vs. 1.5 L/kg in mice (IV); renal clearance was 3.5 times GFR in humans vs. 4 times in mice (Glucophage (metformin hydrochloride) prescribing information, 2009). In both mice and humans, hepatic distribution of metformin is largely dependent on OCT1/Oct1 uptake (Wang et al., 2002; Shu et al., 2007). At a gross pharmacodynamic level, metformin elicits its distinct hepatic, peripheral, and intestinal effects in rodents with progressively greater blood glucose lowering with the route of metformin administration: intravenous infusion < portal infusion < oral administration (Stepensky et al., 2002). More mechanistic studies in mice demonstrated that this species is representative of human pharmacology in that metformin decreases murine intestinal glucose absorption (Wilcock and Bailey, 1991), inhibits murine hepatic gluconeogenesis (Shu et al., 2007), and promotes murine peripheral glucose uptake and utilization (Wilcock and Bailey, 1990). Thus, mice are reasonably representative of metformin PK/PD in humans.

In the present study, the marked reduction in metformin clearance and distribution in Oct1/2-knockout mice ultimately did not result in impaired pharmacodynamic effects. The present findings are conceptually inconsistent with the presumption that systemic pan-OCT inhibition will affect metformin pharmacology (Glucophage (metformin hydrochloride) prescribing information, 2009). The putative pharmaco- and toxico-dynamic implications of such drug interactions are based on the observed increase in metformin systemic exposure by the equipotent OCT1 and OCT2 inhibitor, cimetidine (Somogyi et al., 1987; Wang et al., 2008; Konig et al., 2011). However, cimetidine concentrations sufficient to inhibit metformin renal

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clearance would also be expected to inhibit hepatic uptake, such that the net product of increased systemic metformin concentrations and decreased hepatic uptake would yield little net change in hepatic drug exposure ($dX_{Liver}/dt = C_{systemic} \times CL_{uptake}$). Although metformin hepatic concentrations are not available clinically, notably cimetidine did not affect metformin lactic acidosis; the blood lactate/pyruvate ratio was comparable at two out of four time points tested, was increased and then decreased to a similar extent at the other two times (Somogyi et al., 1987). The concern of enhanced metformin effects due to inhibition of renal clearance and subsequent increase in systemic exposure would be more relevant for a selective OCT2 inhibitor, for example amantadine or amphetamine exhibit 20 fold greater selectivity for OCT2 over OCT1 (Amphoux et al., 2006).

Despite major changes in metformin clearance and distribution in Oct1/Oct2 double-knockout mice, hepatic drug exposure was not reduced to an appreciable extent, and metformin pharmacodynamic effects were not diminished. These findings challenge the presumption that impairment of both renal and hepatic OCT transport is expected to result in altered metformin pharmacodynamics. Furthermore, present studies provided additional evidence that Oct1 is not the sole mechanism of metformin hepatic uptake necessary for inhibition of gluconeogenesis.

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Authorship Contributions:

Participated in research design: Higgins, Bedwell, Zamek-Gliszczynski

Contributed new reagents: N/A

Conducted experiments: Bedwell, Higgins

Performed data analysis: Higgins, Bedwell, Zamek-Gliszczynski

Wrote or contributed to the writing of the manuscript: Higgins, Bedwell, Zamek-Gliszczynski

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transport of 1-(2-methoxyethyl)-2-methyl-4,9-dioxo-3-(pyrazin-2-ylmethyl)- 4,9-dihydro-1H-naphtho[2,3-d]imidazolium bromide (YM155 monobromide), a novel small molecule survivin suppressant. *Drug Metab Dispos* **38:**1-4.

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Figure Legends:

Figure 1. Tetraethylammonium (TEA) plasma concentration-time profiles in wild-type (closed symbols) and Oct1/2-knockout (open symbols) mice following administration of a 10-mg/kg TEA IV bolus dose. Concentration-time profiles are a composite of three groups of four mice, n = 4 mice/group/time point; mean ± S.E.M., *$p < 0.05$ knockout vs. wild-type mice; where error bars are not visible, they are contained within the symbol.

Figure 2. Metformin plasma concentration-time profiles in wild-type (closed symbols) and Oct1/2-knockout (open symbols) mice following administration of metformin as (A) a 5-mg/kg IV bolus dose, or (B) a 10-mg/kg oral dose. Metformin (C) composite plasma $AUC_{0\text{-}2.5hr}$-dose relationship and (D) Cmax-dose relationship over the tested 10-300-mg/kg oral dose range. Concentration-time profiles are a composite of three groups of four mice, n = 4 mice/group/time point; mean ± S.E.M.., *$p < 0.05$ knockout vs. wild-type mice; where error bars are not visible, they are contained within the symbol. Plasma $AUC_{0\text{-}2.5hr}$ values were calculated using the mean concentrations in composite PK datasets, so variability and p values were not calculated.

Figure 3. Metformin liver partitioning in wild-type (closed symbols) and Oct1/2-knockout (open symbols) mice at (A) 1.5 hr, (B) 2 hr, and (C) 2.5 hr post oral administration of metformin. Mean ± S.E.M., n = 3-4 mice/group/data point, *$p < 0.05$ knockout vs. wild-type mice; where error bars are not visible, they are contained within the symbol. (D) Box plot summarizing metformin liver-to-plasma concentration ratios in wild-type and Oct1/2-knockout mice showing 10^{th}, 25^{th}, median, 75^{th}, and 90^{th} percentiles; n = 58-60/group.

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Figure 4. Metformin kidney partitioning in wild-type (closed symbols) and Oct1/2-knockout (open symbols) mice at (A) 1.5 hr, (B) 2 hr, and (C) 2.5 hr post oral administration of metformin. Mean ± S.E.M., n = 3-4 mice/group/data point, *$p < 0.05$; where error bars are not visible, they are contained within the symbol. (D) Box plot of metformin kidney-to-plasma concentration ratios in wild-type and Oct1/2-knockout mice showing 10^{th}, 25^{th}, median, 75^{th}, and 90^{th} percentiles; n = 57-59/group.

Figure 5. Metformin (A) liver and (B) kidney exposure-dose relationship in wild-type (closed symbols) and Oct1/2-knockout (open symbols) mice. Note that tissue $AUC_{1.5\text{-}2.5hr}$ values are presented only for comparison between wild-type and knockout mice and should not be directly related to plasma $AUC_{0\text{-}2.5hr}$ values, which were calculated from the time of dose administration. Composite tissue $AUC_{1.5\text{-}2.5hr}$ values were calculated using the mean concentrations in composite PK datasets (n = 3-4 mice/group/time point), so variability and p values were not calculated.

Figure 6. Effect of metformin on blood glucose levels in wild-type (closed symbols) and Oct1/2-knockout (open symbols) mice following an oral glucose tolerance test. Blood glucose concentration-time profiles in the absence (●,○) or presence (▲,Δ) of metformin administered orally at (A) 10 mg/kg (lowest dose level) and (B) 300 mg/kg (highest dose level). Mean ± S.E.M., n = 10-12, †$p < 0.05$ metformin vs. vehicle within wild-type mice, ‡ $p < 0.05$ metformin vs. vehicle within knockout mice. Blood glucose AUC lowering by metformin as a function of (C) metformin dose and (D) metformin systemic exposure. Mean ± S.E.M., n = 10-12, *$p < 0.05$ knockout vs. wild-type mice.

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Figure 7. Effect of metformin on plasma insulin levels in wild-type (closed symbols) and Oct1/2-knockout (open symbols) mice. (A) Sample plasma insulin concentration-time profiles in the absence (●,○) or presence (▲,Δ) of metformin (PO 30 mg/kg). Mean ± S.E.M., n = 6-12, no statistically-significant differences were observed between groups. (B) Change in plasma insulin AUC in the presence vs. absence of metformin as a function of metformin oral dose. Mean ± S.E.M., n = 6-12, *$p < 0.05$ knockout vs. wild-type mice.

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Tables:

Table 1. Tetraethylammonium (TEA) pharmacokinetic parameters.

Parameter	Wild Type	Oct1/2 Knockout
IV Dose (mg/kg)[a]	**10**	**10**
AUC_{0-t} (μg*hr/mL)	2.41	9.10
$AUC_{0-\infty}$ (μg*hr/mL)	2.84	9.84
C_0 (μg /mL)	22.7	67.4
CL (mL/min/kg)	58.7	16.9
$V_{d,ss}$ (mL/kg)	1520	318
$T_{1/2}$ (hr)	0.31	0.20
Urinary Excretion (% Dose)[b]	35 ± 4	30 ± 6
Creatinine Renal CL (mL/min)	0.09 ± 0.01	0.08 ± 0.01
Inulin Renal CL (mL/min)	0.12 ± 0.03	0.19 ± 0.06
Corrected Recovery (% Dose)[c]	95 ± 11	62 ± 12
TEA renal CL/GFR Ratio	5	1

[a]Noncompartmental parameters were calculated using mean concentrations in composite PK datasets, so variability and p values were not calculated.

[b]Urinary excretion was measured in a separate set of mice with 4 metabolic cages each containing 3 mice per group; mean ± S.E.M. for n = 4 metabolic cages, no statistically-significant differences were observed between groups.

[c]Urinary recovery was corrected based on the ratio of nominal-to-observed murine GFR (Bingham and Cummings, 1985). Nominal murine GFR is 0.28 mL/min (Davies and Morris, 1993), and observed GFR was measured by creatinine and inulin renal clearance.

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Table 2. Metformin pharmacokinetic parameters.

Parameter	Wild Type	Oct1/2 Knockout
IV Dose (mg/kg)[a]	**5**	**5**
AUC_{0-t} (μg*hr/mL)	0.991	4.39
$AUC_{0-\infty}$ (μg*hr/mL)	1.02	4.64
C_0 (μg /mL)	6.25	23.5
CL (mL/min/kg)	81.7	18.0
$V_{d,ss}$ (mL/kg)	1840	530
$T_{1/2}$ (hr)	0.50	0.62
Urinary Excretion (% Dose)[b]	18 ± 2	14 ± 2
Creatinine Renal CL (mL/min)	0.09 ± 0.01	0.08 ± 0.01
Inulin Renal CL (mL/min)	0.12 ± 0.03	0.19 ± 0.06
Corrected Recovery (% Dose)[c]	49 ± 5	29 ± 4
Renal CL/GFR Ratio	4	1
PO Dose (mg/kg)[d]	**10, 30, 60, 100, 300**	**10, 30, 60, 100, 300**
F (%)	59 ± 11	64 ± 4
Tmax (hr)	1.4 ± 0.5	1.0 ± 0.3

[a]Noncompartmental parameters were calculated using mean concentrations in composite PK datasets, so variability and p values were not calculated.

[b]Urinary excretion was measured in a separate set of mice with 4 metabolic cages each containing 3 mice per group; mean ± S.E.M. for n = 4 metabolic cages, no statistically-significant differences were observed between groups.

[c]Urinary recovery was corrected based on the ratio of nominal-to-observed murine GFR (Bingham and Cummings, 1985). Nominal murine GFR is 0.28 mL/min (Davies and Morris, 1993), and observed GFR was measured by creatinine and inulin renal clearance.

[d]Oral bioavailability and Tmax values are presented as the mean ± S.E.M. of the 5 oral dose levels; no dose-dependent trends in bioavailability or Tmax were observed in the 10-300-mg/kg dose range. Individual bioavailabilities at each dose level were calculated using systemic AUC values based on the mean concentrations in composite IV and PO PK datasets (n = 4 mice/group/time point). No statistically-significant differences were observed between groups.

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Figure 1.

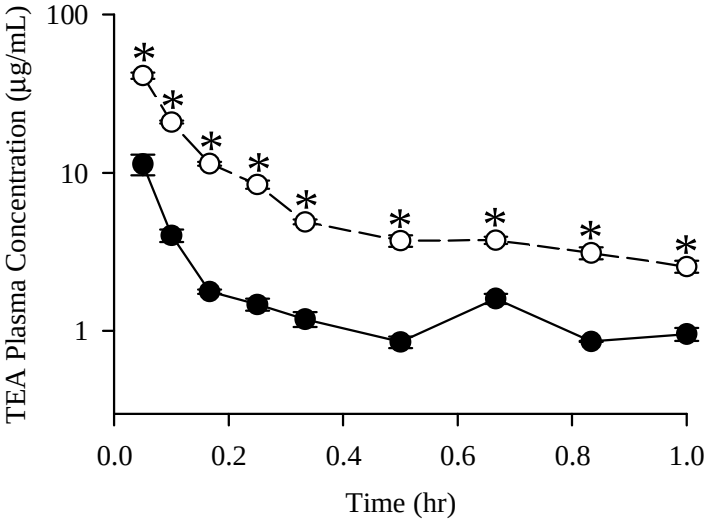

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Figure 2.

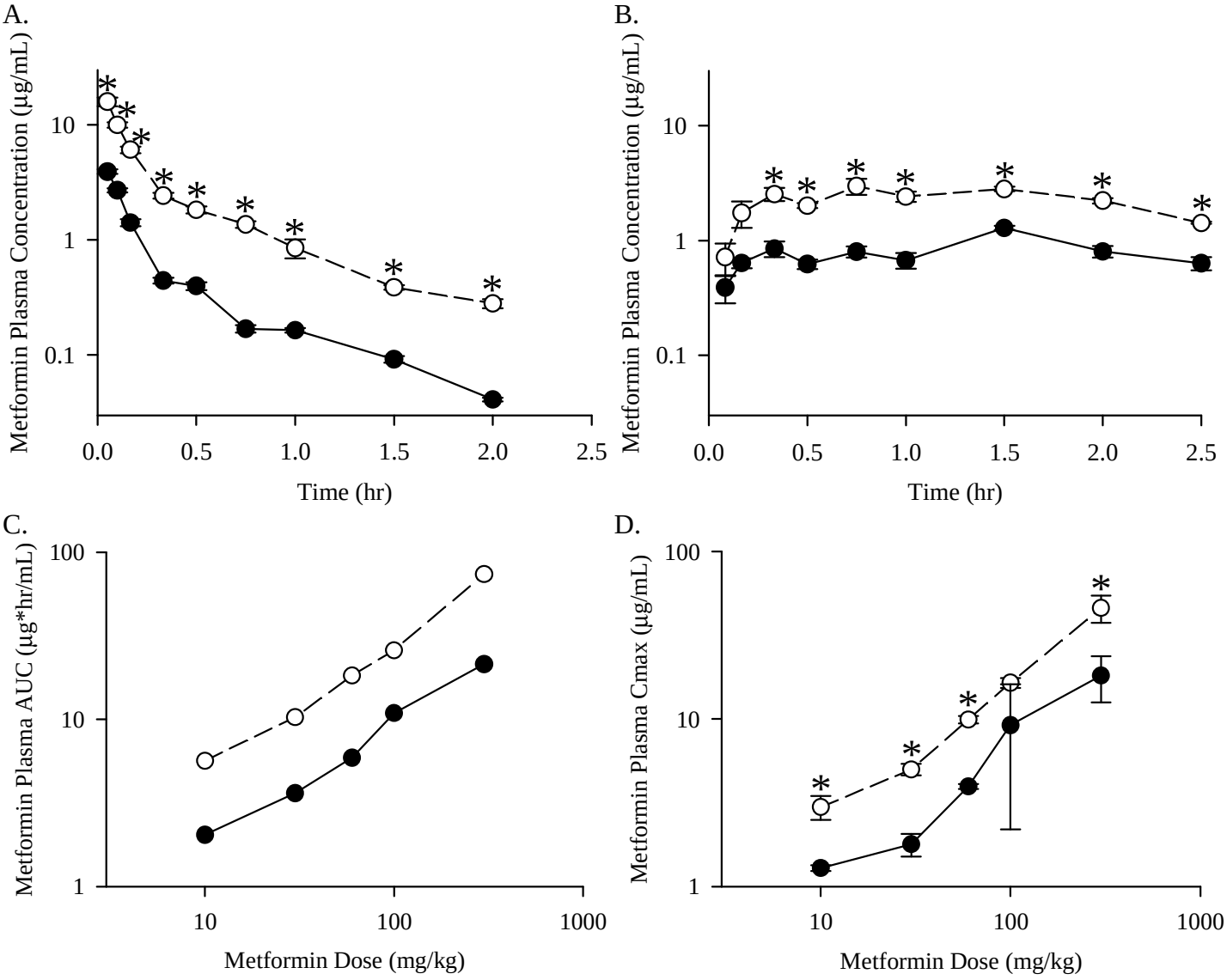

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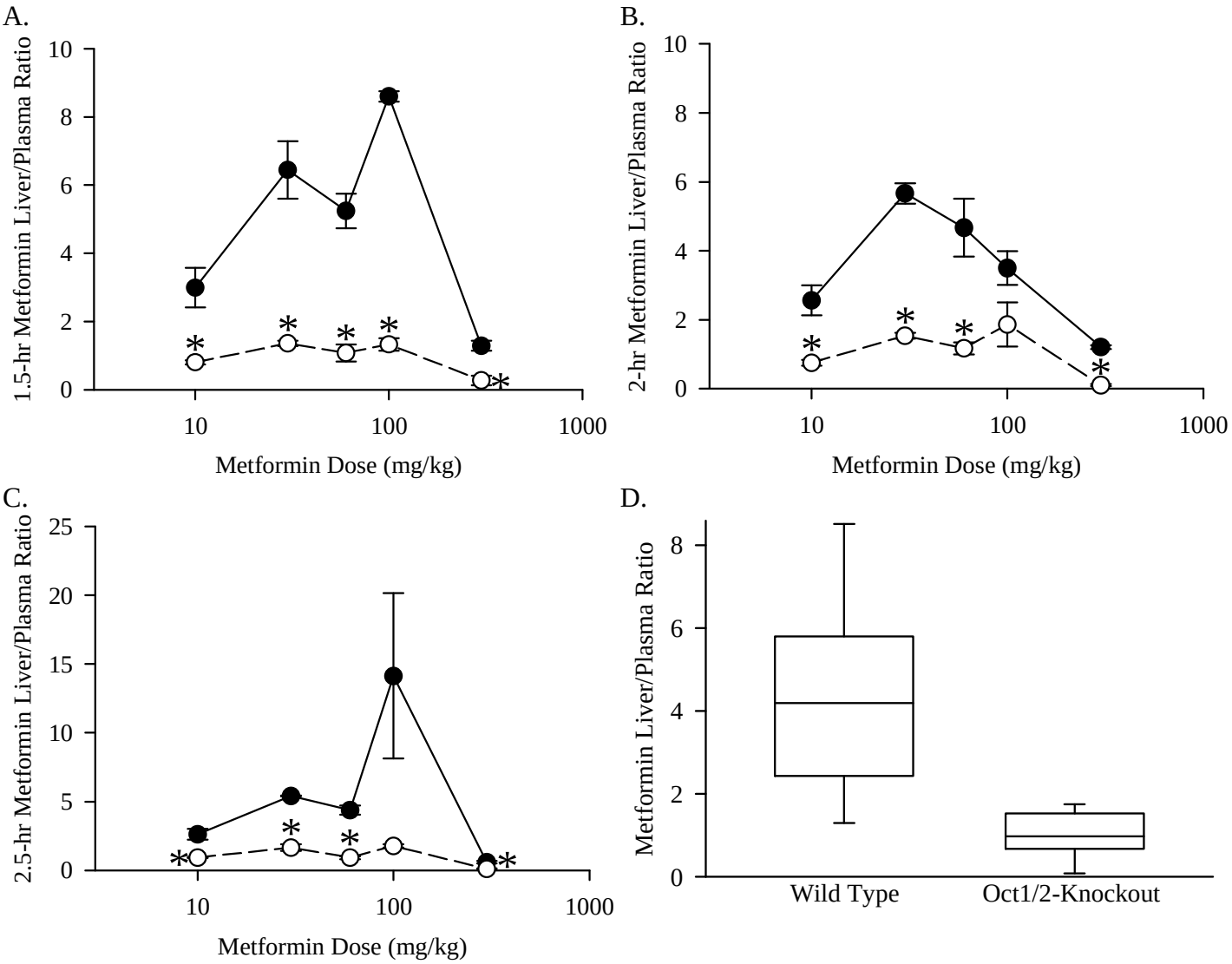
Figure 3.



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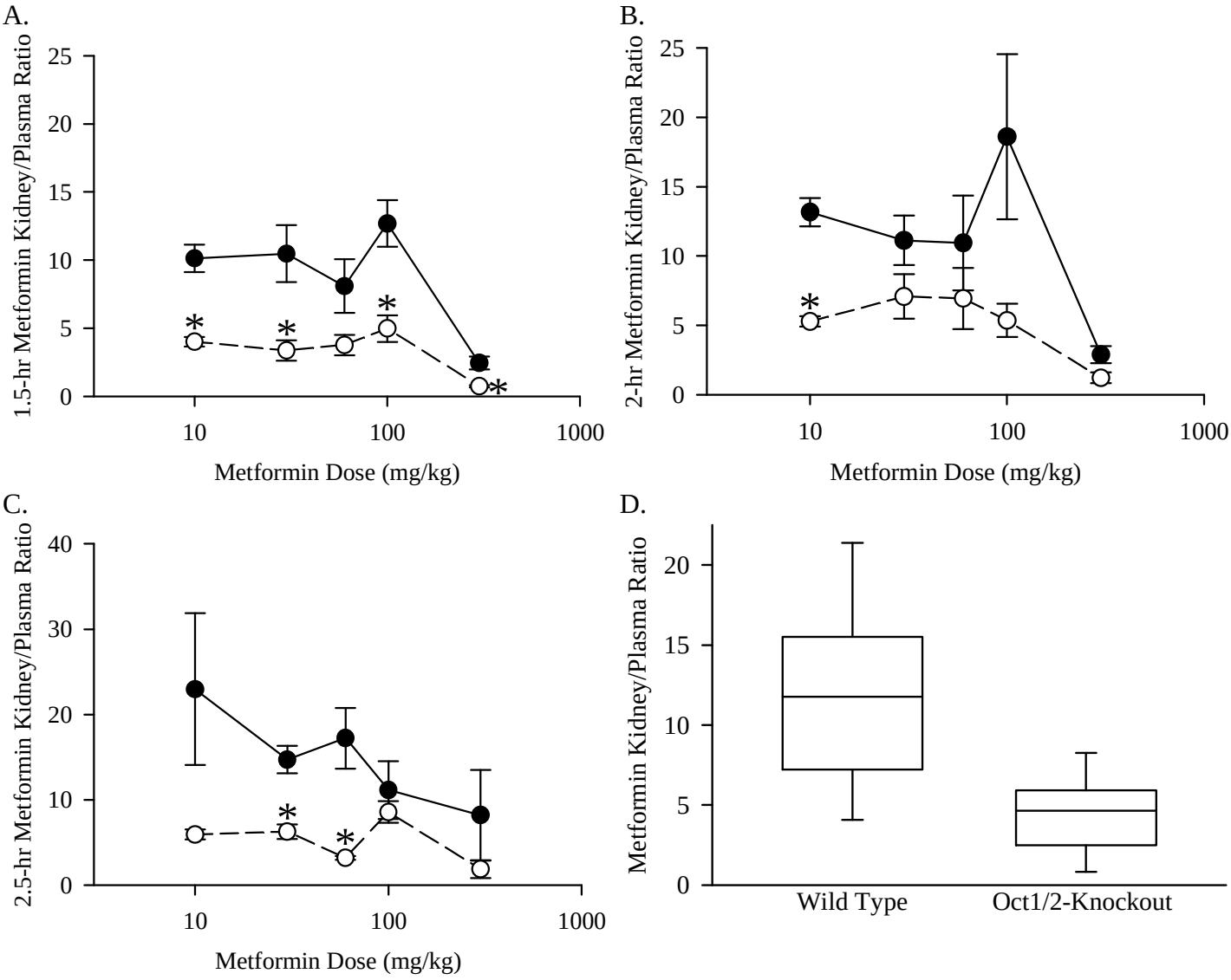

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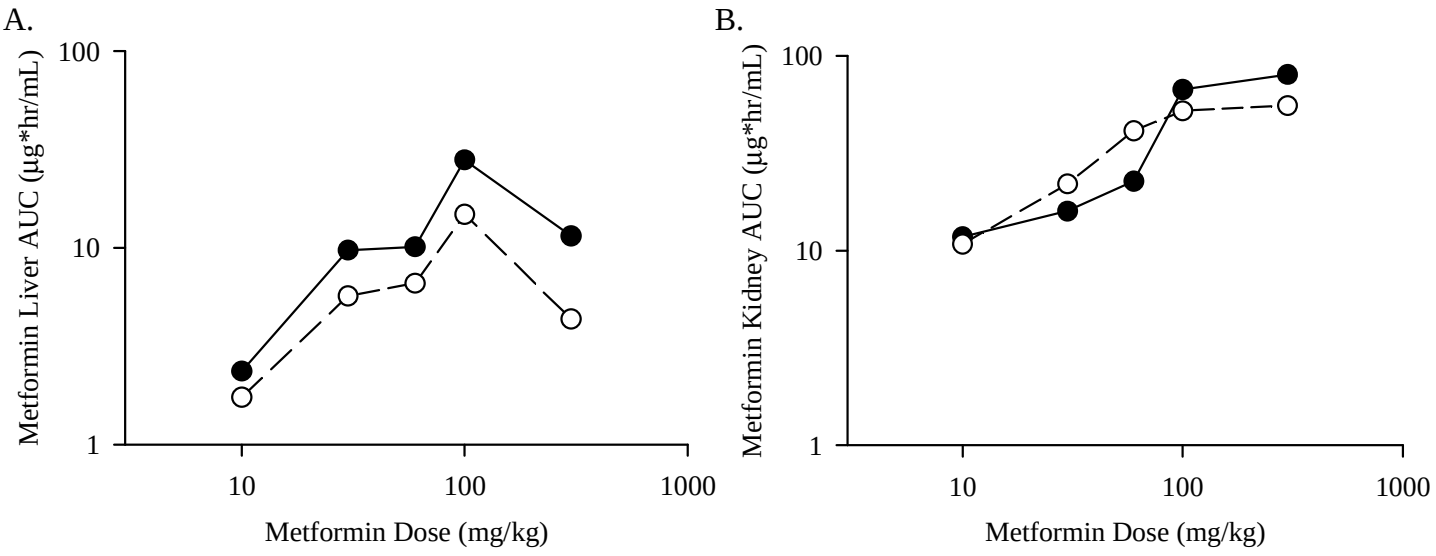

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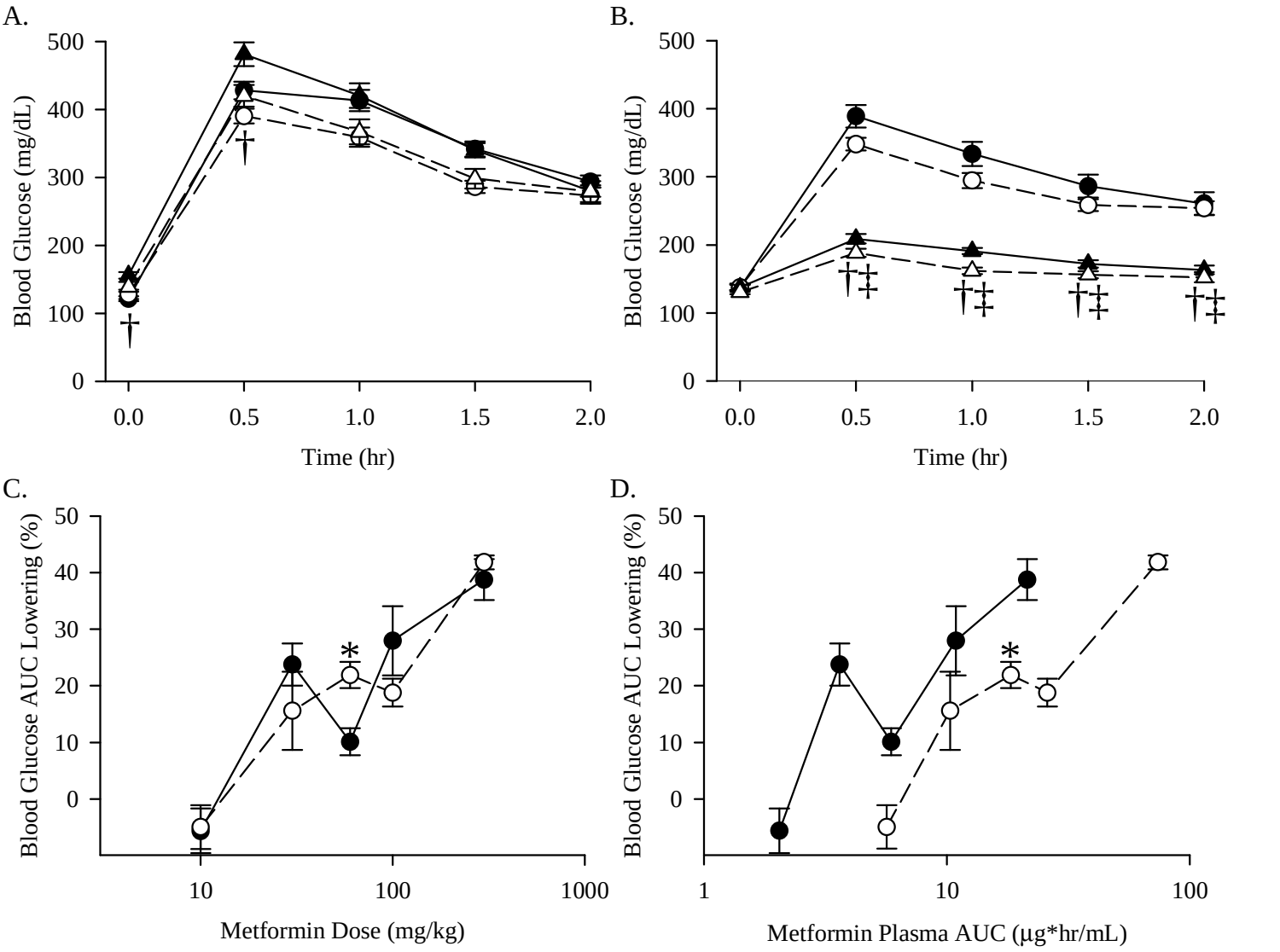

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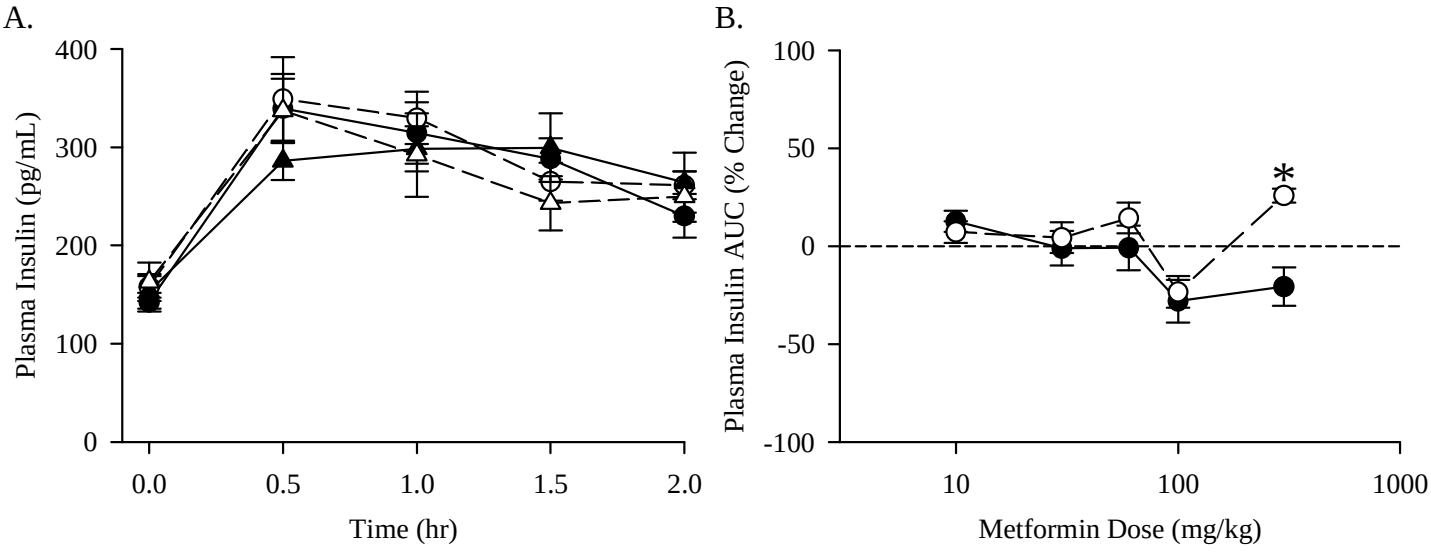

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