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Pharmacokinetics of metformin in the rat: Assessment of the effect of hyperlipidemia and evidence for its metabolism to guanyurea

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Abstract

Metformin pharmacokinetics are highly dependent upon organic cationic transporters. There is evidence of a change in its renal clearance in hyperlipidemic obese patients, and no information on its metabolic fate. To study some of these aspects, the influence of poloxamer 407 (P407)-induced hyperlipidemia on metformin pharmacokinetics was assessed. Control and P407 treated adult male rats were administered 30 mg/kg metformin intravenously (iv). The pharmacokinetic assessments were performed at 2 time points, 36 and 108 *h*, following the intraperitoneal dose of P407 (1 g/kg). mRNA and protein expressions of cationic drug transporters were also measured. There was no evidence of a change in metformin pharmacokinetics after iv doses as a consequence of short term hyperlipidemia, and a change in transporter mRNA but not protein expression in the 108 *h* post-P407 treated rats. Urinary recovery of unchanged drug was high (> 90%) but incomplete. Presumed metabolite peaks were detected in chromatograms of hepatocytes and microsomal protein spiked with metformin. Comparative chromatographic elution times and mass spectra suggested that one of the predominant metabolites was guanyluarea. Hyperlipidemia by itself did not affect the pharmacokinetics of metformin. Guanyluarea is a putative metabolite of metformin in rats.

Introduction

Lipoproteins are involved in the movement mobilization of lipids (triglycerides, cholesterol and phospholipids) between tissues and the blood, which serves as a conduit (Wasan et al. 2008). Hyperlipidemia (HL), a condition of abnormally high concentrations of serum lower density lipoproteins, is a key factor in the development of atherosclerosis and cardiovascular disease. In recent years, the impact of HL on the pharmacokinetic behavior of drugs has been assessed. Certain drugs, in particular those possessing higher log P values, have the capability to sequester into or bind to lipoproteins. Thus HL can be associated with increased plasma binding and lower unbound fraction in plasma, with a reduction in overall drug volume of distribution (Vd) and possibly total body clearance (CL). In certain tissues, however, such binding has been observed for some drug as an increased tissue penetration, which has been attributed to the presence of lipoprotein receptors in those tissues and selective tissue uptake of drug-laden lipoproteins (Patel et al. 2009; Shayeganpour et al. 2008) and/or decrease in efflux transporter expression (Brocks et al. 2014). HL has also been associated with a decrease in expression and function of some cytochrome P450 (CYP) isoforms known to be involved in drug metabolism (Patel and Brocks 2009).

Metformin (MET) is an important treatment choice for type 2 diabetes, a disease associated with other hallmarks of cardiometabolic syndrome including obesity and HL. Metformin disposition in terms of absorption, distribution and renal excretion is highly dependent upon organic cation transport proteins. With respect to its elimination, MET is considered to be mostly excreted unchanged in the urine, although the precise amount has been the subject of some debate with ranges of 80 to 100% having been reported (Graham et al. 2011). The renal clearance (CL_r) has been reported to be approximately 500 mL/min in mostly lean humans. However, in a surgically

untreated group of obese patients given metformin orally, the CL_r was estimated to be considerably lower (337 mL/min), and significantly lower than that in obese patients several months following bariatric surgery (461 mL/min) (Padwal et al. 2011).

This raises a question as to what might lead to a reduced metformin renal CL in obesity. The bariatric surgery patients in the previous study were age, gender and weight matched to the untreated obese group at the time of the metformin pharmacokinetic assessment. However there was one notable difference between the two patient groups, in that the surgically untreated obese patients had significantly higher concentrations of serum triglyceride and total cholesterol than did the bariatric surgical patients. Given the known influence of HL on drug disposition, this raises a question as to whether increased levels of lipoproteins can affect the transporters involved in the disposition of MET.

Obesity is a complex disease state that may be associated with inflammation and other conditions such as diabetes and cardiovascular disease in addition to hyperlipidemia. The single dose poloxamer 407 (P407) induced model of HL is an appropriate choice to assess for the influence of hyperlipidemia on the pharmacokinetics of drugs, as it is non-inflammatory and is not associated with cardiovascular disruption or diabetes. For this reason, we adopted its use to see if, like has been established for CYP, it could affect the solute carrier (SLC) proteins involved in metformin renal excretion and pharmacokinetics. Furthermore because there has been some debate over the extent of MET that is subject to urinary excretion (Graham et al. 2011), a special focus was placed on the urinary recovery of the drug in normolipidemic (NL) rats and the possible metabolic fate of MET in the rat.

Methods

Chemicals

Metformin HCl (>97% purity) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Nicotinamide adenine dinucleotide phosphate tetrasodium (NADPH), P407, fetal calf serum, collagenase, trypsin inhibitor, Percoll gradient, collagen, HEPES sodium salt, bovine serum albumin (BSA) and other general laboratory chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Penicillin-streptomycin, insulin, dexamethasone phosphate, DME media, and trypsin were obtained from GIBCO, Invitrogen Corporation (Carlsbad, CA, USA). Heparin sodium injection, 1000 U/mL and 10000 U/mL, were obtained from Leo Pharma Inc. (Thornhill, ON, Canada). TRIzol reagent was purchased from Invitrogen (Carlsbad, CA). High-Capacity cDNA Reverse Transcription Kit, SYBR Green SuperMix were purchased from Applied Biosystems (Foster City, CA). Real-time PCR primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA) according to previously published sequences.

Animals and induction of HL

All study protocols involving rats were approved by the Institutional Animal Policy and Welfare Committee. Male Sprague–Dawley rats (Charles River, Quebec, Canada) with body weight ranging from 280 to 380 g (2-3 months age) were used. All rats were housed in temperature-controlled rooms with 12 h light per day. The animals were fed a standard rodent chow containing 4.5% fat (Lab Diet® 5001, PMI nutrition LLC, Brentwood, MO). Free access to food and water was permitted prior to the experiments.

The HL rats were injected 1 g P407/kg intraperitoneally (ip) as previously described under light isoflurane anesthesia. The NL control rats were injected equivalent volumes of normal saline. Metformin was administered 36 h later, or at 108 h to represent a longer duration of HL.

Surgery, MET dosing and sample collection

The rats were allocated into three groups, saline-treated NL (controls), acute high lipoprotein concentration of short duration HL (36 *h* after P407), and more sustained HL (108 *h* after P407). The NL control group included 6 rats, whereas HL groups contained 6 rats for the short term HL and 8 rats for the longer term HL. The day before the experiment, the jugular veins of the rats were catheterized with a cannula under isoflurane anesthesia delivered by anesthetic machine as previously described (Hamdy and Brocks 2011). The cannula was filled with 100 u/mL heparin in saline then capped. After recovery, each rat was allowed to have free access to water but not food. The morning after surgery each rat was transferred to a metabolic cage (to facilitate sampling and urine collection) and after adaptation for approximately 30 min with free allowance to water, the rats were administered the intravenous (iv) doses of MET. Food was reintroduced *ad libitum* 2 *h* after the dose was administered.

The maximum human oral dose of MET is 2550 mg (~36 mg/kg/*d*) per day orally (Setter et al. 2003). Hence the metformin single dose level was chosen to be 30 mg/kg. The jv route was used to afford 100% bioavailability, such that cumulative urinary excretion could be determined. The MET injectable solution was prepared in sterile saline for injection to provide a final concentration of 12.5 mg/mL. Each dose was injected over 60 s via the jugular vein cannula, followed immediately by injection of 0.5 mL of saline. Serial blood samples (0.15-0.25 mL) were collected at 0.08, 0.33, 0.65, 1, 2, 3, 4, 6, 8, 12 and 24 *h* post-dose into polypropylene micro-centrifuge tubes. For the first sample, an initial 200 µL volume of blood was discarded followed by the collection of the sample for analysis. Heparin in normal saline (100 U/mL) was used to flush the cannula after each collection of blood. Plasma was separated by centrifugation of the blood at 2500 *g* for 3 min. Urine was collected at the periods 0-3, 3-6, 6-12 and 12-24 *h*,

volume measured and an aliquot saved. All samples were kept at -20°C until assayed for MET using a validated high performance liquid chromatographic method (Gabr et al, 2010). The concentration of potassium phosphate used in the mobile phase was 25 mM.

Effect of HL expression of MET-related urinary excretion transporters

Kidneys were collected from a satellite group of control rats, and rats 36 or 108 h after P407 injection under anesthesia. The tissue samples were directly frozen in liquid nitrogen and kept at -80°C until analysed for mRNA and protein.

Real time PCR

Total RNA was isolated from the frozen kidney tissues using TRIzol reagent (Invitrogen) according to the manufacturer's instructions and quantified by measuring the absorbance at 260 nm. First strand cDNA synthesis was performed by using the High-Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City CA) according to the manufacturer's instructions.

Quantitative analysis of mRNA expression was performed by real-time polymerase chain reaction by subjecting the resulting cDNA to PCR amplification in 96-well optical reaction plates using the ABI Prism 7500 System (Applied Biosystems). The 25 μL reaction mix contained 0.1 μL of 10 μM forward primer, 0.1 μL of 10 μM reverse primer, 12.5 μL of SYBR Green Universal Mastermix, 11.05 μL of nuclease-free water and 1.25 μL of cDNA sample. Sense and anti-sense primer sequences for *Oct1*, *Oct2*, *Mate1* and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) are provided in Table 1). The thermocycling conditions were initiated at

95°C for 10 min, followed by 40 PCR cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. Melting curve for the dissociation stage was performed by the end of each cycle to ascertain the specificity of the primers and the purity of the final PCR product.

The real-time PCR data were analyzed using the relative gene expression (i.e., $\Delta\Delta$ CT) method, as described in Applied Biosystems User Bulletin No. 2. The data are presented as the fold-change in gene expression normalized to the endogenous reference gene GAPDH and relative to a calibrator. The mRNA from NL rats was used as the calibrator to measure the relative change in gene expression caused by HL.

Protein separation and Western blot analysis

Rat kidney biopsies (200 mg) were homogenized in 1 mL of homogenizing buffer (250 mM sucrose, 10 mM HEPES, and 10 mM Tris-HCL, pH 7.4) containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF) using electric tissue grinder. Differential centrifugation was used to obtain a crude membrane fraction. In brief, the kidney homogenates were centrifuged at 9000 g for 10 min at 4°C. Then, the supernatant was spun at 33,000 g for 60 min at 4°C. Finally, the resulting pellet was suspended in phosphate-buffered saline containing 0.1 mM PMSF. Samples were then sonicated on ice for 10 s to ensure homogeneity (Naud et al, 2001) (Naud et al. 2011). Protein concentrations were determined, using BSA as a reference protein. Aliquots were stored at -80°C until analysis.

Protein (50 µg) from each rat kidney was denatured by diluting in 2X loading buffer and heating for 20 min at 36°C. Then protein in each denatured sample was separated by 7% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride

(PVDF) membrane. Thereafter, membranes were blocked for 24 h at 4°C in blocking buffer containing 5% skim milk powder and 0.5% (v/v) Tween-20 in tris-buffered saline solution (TBS; 0.15 M sodium chloride, 3 mM KCl, 25 mM Tris–base). Thereafter, the blocking solution was removed and the blots were rinsed three times in a wash buffer (0.1% Tween-20 in Tris-buffered saline). Then, membranes were incubated with primary antibodies overnight at 4°C. The primary antibody solutions were removed and blots were rinsed three times, followed by incubation with secondary antibody for 2 h at room temperature. Rinsing was then applied to remove the extra secondary antibody. Finally, the protein bands were detected using enhanced chemiluminescence. Protein band intensities (relative to β -actin bands intensity) were quantified using Image-J software (National Institutes of Health, Bethesda, MD, <http://rsb.info.nih.gov/ij/>).

Isolated rat hepatocytes

Male NL rats (300–400 g) were housed in cages and provided water and food ad libitum up to the time of hepatocyte isolation. The isolation of hepatocytes was performed as previously described (Brooks et al. 2014) following a two-step liver perfusion procedure. Cells were placed in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% NL rat serum and antibiotics (penicillin/streptomycin, 1%). After viability assessments cells were added at 0.3 million per well in 300 μ L of cell suspension and the plates were incubated for 6 h at 37°C in a humidifier with 95% O₂ and 5% CO₂. After 6 h, the media containing the dead cells was removed and cells were washed twice with 1X PBS. The media was replaced by DMEM without added serum.

A 10 μ L volume of MET stock solution (2.5 μ g/ μ L) in double distilled water was added to 25 mL DMEM to provide a final MET concentration of 1000 ng/mL. Metformin was co-incubated

with hepatocytes at a concentration of 1000 ng/mL alone or with either β -glucuronidase 1200 Roy U/mL or sulfatase 150 Fishman U/mL for 0, 12 and 24 h ($n = 6$ wells for each time point) (Donato et al. 2004). At the various time points, the hepatocyte incubations were terminated by addition of 0.2 mL water. To each well, 30 μ L of internal standard (Gabr et al. 2010) were added then the well contents were collected and kept at -20°C until assayed.

Exposure of MET to isolated rat microsomes

Hepatic microsomal protein from NL male rats were isolated as previously described (El-Sherbeni and El-Kadi 2014). The total protein concentrations were determined using the Lowry method. The microsomal residues were reconstituted with ice cold sucrose to concentrations of 1 mg protein/mL. Incubation was performed in incubation buffer (3 mM MgCl_2 hexahydrate dissolved in 100 mM potassium phosphate buffer pH 7.4) at 37°C in a shaking water bath. One set of tubes contained microsomal protein only. The second set contained microsomal protein with (100 μ g/mL) MET. The third set contained 100 μ g/mL MET alone with media but no protein. Each set consisted of five tubes. The total volume of microsomal incubates was 1 mL/tube of the buffer. The reaction was initiated by the addition of 1 mM NADPH (final concentration 2 mM) for 30 min at 37°C in a shaking water bath, after which the reaction was terminated by the addition of 300 μ L ice-cold acidified acetonitrile. Aliquots of 100 μ L were subjected to extraction and analysis by HPLC-UV.

In another experiment, MET was incubated with 1 mg/mL microsomal protein from pooled male rat liver microsomes for 0, 20 or 45 min, with the same conditions as expressed above. The reaction was initiated by the addition of 2 mM NADPH (final concentration) and terminated by the addition of 600 μ L ethanol to the 300 μ L incubation mixture. The samples were centrifuged

at 14,000 rpm for 15 min at 0°C. Thereafter, the supernatants were evaporated to dryness and, then, reconstituted in 100 µL of 20% ammonium hydroxide in water and subjected to LC-MS.

Separation and collection of presumed metabolite from microsomal incubates

Based on the chromatographic pattern of microsomal incubates a fractional separation was performed to isolate the chromatographic peak of interest using repeated HPLC injections. Media from the microsomal incubations was extracted (Gabr et al. 2010). To afford separation and allow for subsequent mass spectrometric analysis, reverse phase chromatography was performed using a modified mobile phase that consisted of acetonitrile and water (17:83) adjusted to pH 6.5 using ammonium hydroxide. The column, flow rate and detection wavelengths were as for the regular assay (Gabr et al. 2010). Solution of structurally similar compounds to MET, guanylurea and biuret, were also injected as possible metabolic products.

Mass spectrum characterization

The targeted eluent fraction corresponding to the main presumed metabolite formed from the 0.1 mg/mL MET-microsomal incubations was collected from several HPLC runs. The pooled specimens were vacuum concentrated at room temperature, then and subjected to mass spectrometry. The mass analysis was carried out using a single quadrupole system (Waters Micromass ZQ™ 4000 spectrometer equipped with electron spray ionization (ESI) source. The mass detection was through a direct infusion of the collected eluent and pure 2 µg/mL analyte standard solutions into the detector. Each of the peaks was characterized using selective ion recorder (SIR) in positive ion mode. The other parameter settings were gas source temperature of 140°C, capillary voltage of 3.45 kV and cone voltage of 15 V. The gas flow of desolvation and

cone gas flow were set at 550 and 120 L/h, respectively. These conditions were found to be optimal for ionization of MET, guanylurea and biuret.

The separation of metformin and its metabolite from the 1 mg/mL MET-microsomal incubation was also directly performed using LC-ESI-MS. The compounds were eluted with a mobile phase of 10% acetonitrile in water with 40 mM ammonium carbonate (pH 8) at a flow rate of 0.2 mL/min, using a C18 column (Alltima HP, 250 × 2.1 mm). The mass spectrometer was run in positive ionization mode with an ion scan from m/z of 100 to 135. The nebulizer gas was acquired from an in house high purity nitrogen source. The temperature of the source was set at 150°C, and the capillary and cone voltage were 3.5 kV and 20 V, respectively.

Pharmacokinetic and statistical analysis

Non-compartmental methods were used to characterize the pharmacokinetic properties. The terminal elimination rate constant (λ_z) was calculated by subjecting the plasma concentrations in the terminal phase to linear regression analysis. The $t_{1/2}$ was calculated by dividing 0.693 by λ_z . The AUC_{0-24} of MET (up to 24 h post dose) was calculated using the combined linear and log-linear trapezoidal rule and this was added to the last measured concentration divided by λ_z to determine $AUC_{0-\infty}$. The concentration at time 0 h after iv dosing was estimated by back extrapolation of the initial log-transformed concentrations to time 0. The total CL was calculated as the quotient of dose to $AUC_{0-\infty}$ and the steady state V_d (V_{dss}) as $CL \times AUMC / AUC$, where AUMC is the area under the first moment plasma concentration vs. time curve, from time of dosing to infinity. The mean residence time (MRT) was determined as the quotient of AUMC to AUC. The CL_r was calculated as the product of urinary recovery and CL, and nonrenal CL (CL_{nr}) as the difference of CL_r from CL. Between-group differences in the arithmetic means of

continuous baseline variables were analyzed using paired Student's *t*-test (if the normality assumption passed) or a Mann-Whitney test (if the normality assumption failed).

All compiled data were expressed as mean $\pm$ SD unless otherwise indicated. Student's *t*-test were performed using Microsoft Excel 2013 (Redmond WA). The level of significance in all statistical testing was set at $\alpha = 0.05$. Bonferroni correction was applied to the multiple comparisons between pharmacokinetic parameters of control and HL groups.

Results

After iv administration of 30 mg/kg of MET, HL was found not to cause any significant change in MET pharmacokinetics whether the dose was administered at 36 or 108 *h* from the time of injection of P407 (Table 2, Figure 1). This was reflected in the pharmacokinetic parameters, none of which was found to differ from the NL group. Two extra rats were included in the HL 108 *h* group of rats, because there was some loss of urine during collection (those rats were excluded from CL_r calculations). It was found that from 92 to 100% of the MET dose was eliminated unchanged via urinary excretion, and CL_r accordingly accounted for the majority of the measured CL in all groups.

Expression of rat OCT1, OCT2 and MATE1 transporters in kidney

There were no significant differences noted between control and 36 *h* post-P407 in the measured kidney mRNA of *Oct1*, *Oct2* or *Mate1*. At 108 *h* after injection of P407 there were significant decreases in mRNA of 48% and 66%, respectively, for *Oct1* and *Mate1* respectively (Figure 2).

The CV was close to 30% for both test groups in these mRNA. However, HL did not change the mRNA expression of *Oct-2* where the test group had high variability (CV% = 57) (Figure 2).

With respect to protein, although a trend was apparent (each mean being numerically lower in value) for the transporters at 108 h after P407 compared to NL controls, there were no statistically significant differences present in protein concentration of any of the three examined kidney transporters in HL (36 or 108 h) and control animals.

Assessment of MET metabolism

Chromatograms of MET incubated hepatocytes

Immediately after addition of MET into wells containing hepatocytes, there was no appearance of unusual peaks, only the peak conforming to MET at ~11.6 min. However, with time (12 and 24 h) a new peak appeared, which eluted just before MET (at ~10.5 min). In addition, the peak at 4.8 min, which also appeared in the media-only blank sample, was noticeably higher after 12 to 24 h MET-hepatocyte incubations (Figure 3). The incubation of hepatocyte samples with Phase II hydrolyzing enzymes caused no increase in the concentrations of MET.

Incubation of MET with rat microsomal protein

Upon the incubation of 100 µg/mL MET for 30 min with isolated rat microsomes (1 mg protein/mL), two peaks became clearly evident in the chromatograms of extracted samples which were not present in the chromatograms of the blank microsomes or drug injected alone. These coincided with the observations of the peaks noticed in the hepatocyte incubations, except here the peak eluting at 4.8 min became more apparent because it was not present in the blank microsomal mixture.

Subsequently other incubations were extracted and the pooled residues reconstituted in methanol. This was then injected into a modified mobile phase free of LC-MS incompatible additives (to facilitate possible evaporation/concentration, fractional separation by HPLC/UV and subsequent mass characterization). Using the modified mobile phase (Figure 3, middle panel), it was observed that a presumed metabolite peak eluted at nearly the same retention time (4.8 min) as was the case for the mobile phase (Gabr et al. 2010) used in the microsomal and hepatocyte incubation experiments (Figure 3, top and middle panels). Upon direct injection of some compounds closely related to MET with the modified mobile phase, biuret yielded an elution peak (Figure 3, bottom panel) that did not match that of the unknown peak. However, an injection of guanylylurea yielded an elution peak that coincided with that of the unknown peak (Figure 3, bottom panel). Fractional separation was then performed to collect that peak of interest for subsequent mass spectrometric characterization.

Spectral analysis of the primary isolated presumed metabolite

A mass spectral analysis of the isolated eluted peak of interest (at 4.8 min, Figure 3, bottom panel) from the HPLC run was performed. The largest ion had m/z ratio of 103, with a smaller one of m/z 104.9. MET in contrast had an m/z ratio of 129.9. Guanylylurea gave a large ion with m/z ratio of 103, which precisely matched that of the largest m/z ratio in the collected microsomal incubation fraction (Figure 4). This suggested that the main component in the eluent fraction collected from the HPLC injection was guanylylurea.

The smaller peak of 105 m/z suggested the possibility of being a reduced form of guanylylurea, possibly diaminomethylurea. To explore this possibility, the reducing agent, sodium borohydride (40 mg), was added to 2 mL of guanylylurea (10 mg/mL) in absolute ethanol at -20° (Hubert et al.

1975). For the first 6 *h*, 1 μ L of HCl (2 N) was added every 15 min to the reaction mixture, vortexed, then re-incubated at -20°C. After 24 *h*, the excess of sodium borohydride was removed by the addition of 20 μ L concentrated HCl. At 0, 15 min, 30 min, then 1, 2, 3, 6 and 24 *h*, 10 μ L of the reaction mixture was diluted in 1 mL absolute ethanol, and directly infused in the mass spectrometer (Waters Micromass ZQ 4000 spectrometer), using a flow rate of 40 μ L/min, source temperature of 140°C, capillary and cone voltages of 3.5 kV and 20 V, respectively. There was no noticeable appearance of a peak at *m/z* 105 under these reducing conditions.

In the second experiment in which the incubate was injected on a C18 column, the LC-MS chromatogram showed no peak at 5 min in the 0 *h* incubation of MET with microsomal protein. After 30 and 45 min incubation of MET with the microsomal protein and cofactors, there was an appearance and growth of a peak at 5 min in the chromatograms which, as in the HPLC-UV run (Figure 3 bottom panel), coincided with the elution time of guanylylurea (Figure 5).

Discussion

Profound changes can occur in the pharmacokinetics of drugs in the presence of P407-induced HL. Most of these drugs are known to bind extensively (>70%) to plasma proteins (Brocks et al. 2006; Brocks and Wasan 2002; Choi et al. 2014; Kobuchi et al. 2011; Lee et al. 2011, 2012a; Lee et al. 2012c; Shayeganpour et al. 2005; Sugioka et al. 2009). Hyperlipidemia induced by P407 is also known to cause a downregulation in the expression of several CYP isoforms in liver of male rats, including CYP3A1/2 and CYP2C11, and decrease in mRNA of several proteins involved in drug transport and metabolism (Brocks et al. 2013; Shayeganpour et al. 2008). These changes in protein expression may account for alterations in the pharmacokinetics of drugs that

are intrinsically less highly bound (Lee et al. 2012b) or in which the unbound fraction of drug is not affected by the presence of HL (Choi et al. 2014; Lee et al. 2012c).

Metformin is negligibly bound to plasma proteins and minimally metabolized, but is a major substrate for SLC transporters including Oct1/2 and Mate1 isoforms, which are known to be involved in the renal secretion of metformin in rats and humans (Graham et al. 2011; Kimura et al. 2005; Komazawa et al. 2013; Ma et al. 2015). The data show that at 36 h post-P407, the concentrations of serum lipids are at levels far exceeding what would be expected in human serum. By 108 h after administration, those lipid levels are more in line with what might be seen in line with HL patients. In neither case though was P407 associated with a change in MET concentrations. In the 108 h group of rats there was some decrease in the mRNA of *Oct1* and *Mate1*, although no changes were noted in the corresponding protein concentrations (Figure 2). The results might suggest that the apparently lower MET CL_r in obese humans is not due to HL alone, but rather to some other aspect of obesity, perhaps inflammation, which is not associated with the P407 model of HL (Chaudhary and Brocks 2013).

The data raised other issues in relation to MET pharmacokinetics in the rat. Although there has been some uncertainty as to the precise extent of urinary excretion of MET in humans, it is generally considered to be the predominant pathway of elimination with 80-100% recovery being reported, with a minor degree of metabolism (Beckmann 1968; Graham et al. 2011). On the other hand, there have been recent reports suggesting that MET is appreciably metabolized in the rat. In a series of papers it was suggested that only 50-67% (Lee et al. 2008) of an iv dose of MET is excreted via the urine in male Sprague-Dawley rats, values appreciably lower than reported in humans (Choi et al. 2008a,b,c, Choi et al. 2007a,b, 2010a; Choi and Lee 2006, 2012). However some of these papers by the same group of investigators found higher recoveries (78% over 24 h

(Choi et al. 2006) and 79% over 48 h (Choi et al. 2010)) in rat urine, values nearer those reported in humans. In all cases the rats had been administered the same dose of MET, 100 mg/kg. In contrast, in our rats we attained a recovery in urine ranging from 92% to complete (Table 2). The maximum therapeutic dose of MET is 2 g per day, or approximately 30 mg/kg as given to our rats. In contrast, those rats in which the proportion of nonrenal CL being upwards of 50% of total CL were given higher iv doses of 100 mg/kg. Compared to our AUC and estimates of CL_r to those reports using the higher doses there is some suggestion for saturation of CL with increased dose. However, in one of the abovementioned studies comparing 50, 100 and 200 mg/kg doses (Choi et al. 2006) no evidence of saturation in urinary recovery was observed.

Aside from the extent of urinary excretion, based on the results of rat liver and intestinal microsomal incubations it has been demonstrated that MET concentrations could be lowered by apparent metabolism *in vitro* (Choi et al. 2010a,b, Choi and Lee 2012). No evidence of what it is possibly metabolized to in mammals has been published to date, however. In examining some chromatograms from our previous human trial and also from the rats given MET in this study, some small peaks were noticed eluting before MET, that were MET-related because they were not visible in urine of humans not given MET. Some of these same peaks were found in rat hepatocytes exposed to MET and to microsomal protein isolated from NL rat livers (Figure 3).

MET is the most widely used of all antidiabetic drugs and its dose levels are in the gram range, being amongst the highest of all clinically used drugs. Hence, although the percentage of metabolism is reportedly small, even 10 to 20% potential metabolism in humans as has been suggested by some authors, could potentially generate appreciable body exposure to metabolite. Guanylurea was recently reported to be a bacterial breakdown product of MET in sewage (Trautwein and Kummerer 2011). Using the modified mobile phase we found a microsomal

product peak we had fractionally collected from the HPLC column eluent to precisely co-elute with guanylylurea. Further support for guanylylurea as a metabolic candidate was provided in the mass spectrographic patterns where the predominant ion of m/z ratio in the collection fraction matched that of guanylylurea. The LC-MS tracings of injected microsomal incubate and separation through a C18 column further supported the existence of guanylylurea as a metabolite of MET. The possibility of the less prevalent ion at 104.9 m/z seems unlikely to be a reduced microsomal product of guanylylurea because it did not appear when guanylylurea was exposed to the reducing agent NaBH_4 . The toxicological or pharmacological relevance of guanylylurea as a metabolite of metformin is unknown, as there is a lack of data in the literature (ter laak and Baken 2014).

In conclusion, HL induced using a single dose of P407 had no influence on the pharmacokinetics of MET. Although there was some change in mRNA of two transporters involved in MET transport, it did not translate into a change in protein expression within 4.5 d of the dose of P407. Although urinary recovery in the rat was nearly complete after iv doses of 30 mg/kg, we were able to detect the presence of small quantities of MET-related metabolite peaks in chromatograms of urine, hepatocytes and microsomal incubations. Guanylylurea was identified to be a putative metabolite of MET in the rat.

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Legend to figures:

Figure 1: Mean plasma concentration vs. time plots of MET after intravenous administration of 30 mg/kg as iv doses to normolipidemic (NL) or hyperlipidemic (HL) rats.

Figure 2: Effect (mean $\pm$ SD) of HL after 108 *h* ip injection of P 407 on the mRNA and protein expression of rat kidney OCT1, OCT2 and MATE1 (*n* = 4/group). Solid bars indicate control NL rats, cross hatched bars indicate treatment HL rats. Significant differences of HL from NL are denoted by *.

Figure 3. Top panel: Effect of hepatocytes on MET metabolism after exposure to 1000 ng/mL MET. Representative HPLC-UV chromatograms are depicted for a) hepatocyte-free media for 24 *h*, b) hepatocyte-free media containing 1000 ng/mL MET for 24 *h*, and hepatocytes exposed to MET for c) 0 *h*, d) 12 *h*, and e) 24 *h*. Middle panel: Chromatogram depicting a) MET-free microsomal protein, b) MET in buffer without microsomal protein, and c) microsomal incubation with MET 100 μ g/mL for 30 min. Lower panel: Chromatograms showing direct injection of standard solutions of guanyldurea (1 μ g/mL), biuret (1 μ g/mL) and MET (50 ng/mL) and the collected eluent of the peak eluting at 4.8 min in the microsomal incubation using the modified mobile phase. The blank is the extracted microsomal media without addition of MET.

Figure 4: Mass spectra and chemical structures, from top down, MET-metabolite eluent fraction, guanyldurea, biuret and MET. All compounds were dissolved or reconstituted in double distilled water containing 1% formic acid. The major ion of *m/z* 103 matched that of guanyldurea.

Figure 5: Mass spectral chromatograms of injection of MET after exposure to rat microsomal protein for 0, 20 and 45 min (ion scan from *m/z* 100 to 135.). The MET eluted at 10 min under these conditions. A peak appeared with progression of incubation times at 5 min, which corresponded with the elution time of guanyldurea (indicated as control injection containing guanyldurea and metformin standards in the same sample).

Table 1: Sequences of rat primers used for real time PCR analysis

Gene	Sense primer	Anti-sense primer
<i>Oct1</i> (Shi et al. 2008)	TCC TGC TGA CCT GAA GAT GCT	GAA CAG GTC GGC AAA CGA TAG
<i>Oct2</i> (Kristufek et al. 2002)	GCT TGG GTA GAA TGG GCA TC	GTG AGG TTG GTT TGT GTG GG
<i>Mate1</i> (Kajiwara et al. 2007)	CAC ACT GGC AAT TGC GGT TA	CTT CAA GTT CTG GCT CCC GT
<i>Gapdh</i> (Takizawa et al. 2005)	GGC CAA GGT CAT CCA TGA	TCA GTG TAG CCC AGG AGG

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Table 2: Pharmacokinetics (mean±SD) of MET in normolipidemic (NL) and hyperlipidemic (HL) rats given 30 mg/kg of MET intravenously.

Parameter	Treatment group (n)		
	NL (6)	HL 36 h (6)	HL 108 h (8)
$AUC_{0-\infty}$, $\mu\text{g}\cdot\text{h/mL}$	17.7±4.33	13.4±3.75	13.9±2.58
CL , mL/min/kg	29.5±6.02	39.7±9.71	37.2±6.75
CL_r , mL/min/kg	27.2±5.66	36.4±11.5	35.3±5.87 ^a
CL_{nr} , mL/min/kg	2.43±2.02	3.55±5.48	0.0659±0.161 ^a
Vd_{ss} , L/kg	2.32±1.38	2.76±1.22	1.73±0.755
MRT , h	1.26±0.599	1.16±0.440	0.761±0.252
$t_{1/2}$, h	4.88±2.52	3.01±1.12	2.77±0.698
Urinary recovery, %	92.2±7.09	92.2±15.0	102±3.24 ^a
^a based on 6 rats (urine was lost from 2 rats)			

Draft

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Figure 1: Mean plasma concentration vs. time plots of MET after intravenous administration of 30 mg/kg as iv doses to normolipidemic (NL) or hyperlipidemic (HL) rats.

105x104mm (300 x 300 DPI)

Figure 2: Effect (mean $\pm$ SD) of HL after 108 h ip injection of P 407 on the mRNA and protein expression of rat kidney OCT1, OCT2 and MATE1 (n = 4/group). Solid bars indicate control NL rats, cross hatched bars indicate treatment HL rats. Significant differences of HL from NL are denoted by *.

96x77mm (300 x 300 DPI)

Figure 3. Top panel: Effect of hepatocytes on MET metabolism after exposure to 1000 ng/mL MET. Representative HPLC-UV chromatograms are depicted for a) hepatocyte-free media for 24 h, b) hepatocyte-free media containing 1000 ng/mL MET for 24 h, and hepatocytes exposed to MET for c) 0 h, d) 12 h, and e) 24 h. Middle panel: Chromatogram depicting a) MET-free microsomal protein, b) MET in buffer without microsomal protein, and c) microsomal incubation with MET 100 μ g/mL for 30 min. Lower panel: Chromatograms showing direct injection of standard solutions of guanyurea (1 μ g/mL), biuret (1 μ g/mL) and MET (50 ng/mL) and the collected eluent of the peak eluting at 4.8 min in the microsomal incubation using the modified mobile phase. The blank is the extracted microsomal media without addition of MET.

141x237mm (300 x 300 DPI)

Figure 4: Mass spectra and chemical structures, from top down, MET-metabolite eluent fraction, guanylurea, biuret and MET. All compounds were dissolved or reconstituted in double distilled water containing 1% formic acid. The major ion of m/z 103 matched that of guanylurea.

215x148mm (300 x 300 DPI)

Figure 5: Mass spectral chromatograms of injection of MET after exposure to rat microsomal protein for 0, 20 and 45 min (ion scan from m/z 100 to 135.). The MET eluted at 10 min under these conditions. A peak appeared with progression of incubation times at 5 min, which corresponded with the elution time of guanyurea (indicated as control injection containing guanyurea and metformin standards in the same sample).

200x123mm (300 x 300 DPI)