BIOPHARMACEUTICS & DRUG DISPOSITION
Biopharm. Drug Dispos. **30**: 476–484 (2009)
Published online 17 September 2009 in Wiley InterScience
(www.interscience.wiley.com) **DOI**: 10.1002/bdd.684

A Comparison of Uptake of Metformin and Phenformin Mediated by hOCT1 in Human Hepatocytes

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ABSTRACT: Metformin, a biguanide that has been used to treat type 2 diabetes mellitus, is reportedly transported into human hepatocytes by human organic cation transporter 1 (hOCT1). The objective of this study was to investigate differences in the hepatic uptake of metformin and phenformin, a biguanide derivative similar to metformin. Special focus was on the role of active transport into cells. Experiments were therefore performed using human cryopreserved hepatocytes and hOCT1 expressing oocytes. Both biguanides proved to be good substrates for hOCT1. However, phenformin exhibited a much higher affinity and transport activity, with a marked difference in uptake kinetics compared with metformin. Both biguanides were transported actively by hOCT1, with the active transport components much greater than passive transport components in both cases, suggesting that functional changes in hOCT1 might affect the transport of both compounds to the same degree. This study for the first time produced detailed comparative findings for uptake profiles of metformin and phenformin in human hepatocytes and hOCT1 expressing oocytes. It is considered that hOCT1 may not be the only key factor that determines the frequency of metformin and phenformin toxicity, considering the major contribution of this transporter to the total hepatic uptake and comparable width of their therapeutic concentrations.

Key words: human organic cation transporter 1 (hOCT1); metformin; phenformin; hepatocytes; oocytes

Introduction

Metformin, a biguanide derivative, has been used in the treatment of type 2 diabetes for nearly 50 years. The major effects are to reduce hepatic glucose [1,2], increase insulin-stimulated glucose uptake in skeletal muscle and adipocytes [3,4], inhibit intestinal glucose absorption [5] and activate AMPK (AMP-activated protein kinase) in both hepatocytes and skeletal muscle [6].

It was recently reported that human organic cation transporter 1 (hOCT1) is responsible for the hepatic uptake of metformin [7–9]. This factor is expressed primarily in the liver, a major target organ of biguanides [10,11]. Shu *et al*. [12,13] reported that the hOCT1 genotype is a determinant of metformin pharmacokinetics and pharmacodynamics. Wang *et al*. [14] also described decreased metformin in the livers of Oct1 knockout mice with low blood lactate concentrations. These results indicate that hOCT1 plays an important role in hepatic uptake and that the pharmacokinetics of metformin might depend on transporter activity in the liver. Therefore, it is conceivable that hOCT1 is a regulatory factor

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Received 17 December 2008
Revised 30 June 2009
Accepted 16 August 2009

determining the frequency of lactic acidosis with metformin.

Biguanides have the severe side effect of lactic acidosis. Although both metformin and phenformin are biguanide derivatives, there are differences in the frequency at which they induce lactic acidosis. The frequency of phenformin-associated lactic acidosis has been estimated to be 4.0 cases per 10000 treatment years [15]. In contrast, metformin-associated lactic acidosis only occurs in 0.3 cases per 10000 treatment years, lower by 10 to 20 times [16]. The frequency of lactic acidosis caused by metformin is less than that of severe hypoglycemia induced by sulfonylurea [17]. Furthermore, the United Kingdom Prospective Diabetes Study (UKPDS) showed that intensive treatment with metformin significantly reduces macrovascular end-points and mortality in individuals with newly diagnosed type 2 diabetes, compared with intensive treatment with insulin or sulphonylurea derivatives [18]. Thus, metformin is regarded as a safe drug and the first-line drug for the treatment of mature-onset diabetes, that has failed control by diet. However, the reasons for the differences are not clear.

There has not been a detailed study to compare the hepatic uptake characteristics of metformin and phenformin in human hepatocytes. The present study was therefore designed to investigate differences in the hepatic uptake of metformin and phenformin. This report presents uptake profiles for [^{14}C]metformin and [^{14}C]phenformin in cryopreserved human hepatocytes and hOCT1 expressing oocytes.

Materials and Methods

Materials

[^{14}C]Metformin hydrochloride (specific activity, 962 MBq/mmol) was purchased from Moravek Biochemicals (Brea, CA). [^{14}C]Phenformin hydrochloride (specific activity, 4.48 GBq/mmol) was synthesized at Amersham Biosciences UK Ltd (Little Chalfont, UK). Metformin was purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan) and phenformin from Sigma-Aldrich Co. (St Louis, MO). Tetraethylammonium (TEA) bromide was obtained from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan). *p*-Aminohippuric acid (PAH) was purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan).

Cryopreserved human hepatocytes were purchased from Xenotech LLC (Lenexa, KS). Transportocytes of *Xenopus laevis* (African claw frog) expressing human hOCT1 (organic cation transporter-1: *SLC22A1*) were purchased from BD-GENTEST (Woburn, MA).

All other chemicals and reagents used were commercial products of reagent grade.

Preparation of [^{14}C]metformin and [^{14}C]phenformin solutions

Metformin (3.67 mM) and [^{14}C]metformin (0.01 mM) were dissolved in diluted water to give a stock solution of [^{14}C]metformin (3 mM, 3.68 MBq/ml). Phenformin (3.67 mM) and [^{14}C]phenformin (0.01 mM) were dissolved in diluted water as a stock solution of [^{14}C]phenformin (3 mM, 3.68 MBq/ml).

Preparation of human cryopreserved hepatocytes

Six batches of human hepatocytes (lot number 489, 508, 548, 591, H306A and XHT012803) were used. Immediately before the study, the cryopreserved hepatocytes were thawed at 37°C and added to hepatocyte isolation medium (Xenotech LLC), containing DMEM and isotonic percoll, and centrifuged at $70 \times g$ for 5 min at room temperature. The supernatant was aspirated and discarded. The cells were suspended in Krebs-Henseleit buffer (118 mM NaCl, 5 mM KCl, 1.1 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 10 mM glucose and 10 mM HEPES, pH 7.4). After verification of viability by a trypan blue exclusion test, suspensions with a viability of more than 70% were used. Finally, the cells were resuspended in the Krebs-Henseleit buffer to give a final cell density of 2.0×10^6 cells/ml for the uptake study.

Uptake by human cryopreserved hepatocytes

Before the uptake studies, the cell suspensions (2.0×10^6 cells/ml) were preincubated at 37°C for 5 min. Then, uptake reactions were initiated by adding an equal volume of buffer (0.15 ml) containing either [^{14}C]metformin (20, 100, 500, 1000, 2000, 5000 and 10000 μM) or [^{14}C]phenformin

DOI: 10.1002/bdd

(1, 5, 10, 50, 100, 500 and 1000 μM) to the cell suspension. At designated time points (20 min for metformin, 10 min for phenformin), the reaction was terminated by separating the cells from the reaction medium. For this purpose, an aliquot (0.1 ml) of incubation mixture was collected and transferred to a centrifuge tube containing 0.05 ml of 2 N NaOH solution overlaid with silicone oil. Then, the tube was centrifuged at 10000 × *g* for 15 s to precipitate the cells into the alkaline layer through the silicone oil layer to terminate the uptake reaction. After the cells were solubilized in the alkaline layer, the bottom of the tube containing the solubilized cell layer was sliced off with a razor blade, and the contents were transferred to a scintillation vial. After addition of 1 ml of 2 N NaOH and sonication, 5 ml of liquid scintillator was added to the vial and the radioactivity was determined using a liquid scintillation counter. Furthermore, to examine whether substrate uptake by human hepatocytes might be membrane potential-dependent, the cells were incubated with high K^+ buffer (5 mM NaCl, 118 mM KCl, 1.1 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 10 mM glucose and 10 mM HEPES, pH 7.4) instead of Krebs-Henseleit buffer at 37°C for 10 min with 20 μM [^{14}C]metformin or for 5 min with 1 μM [^{14}C]phenformin. For the inhibition study, the cell suspensions were incubated with reaction medium containing unlabelled substrates, metformin (20 mM) and phenformin (1 mM), and inhibitors, PAH (0.1, 1 mM) and TEA (0.1, 1 mM). The other procedures were performed in the same way as in the membrane potential-dependent study.

Uptake by oocytes

Oocytes expressing hOCT1 (10 oocytes/tube) were placed in a test tube with a small volume of modified Barth's medium (110 mM NaCl, 1 mM KCl, 0.41 mM $CaCl_2$, 2.5 mM $NaHCO_3$, 1 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 7.5 mM Tris-HCl, pH 7.4). Then, the modified Barth's medium was removed and replaced with 0.3 ml of Na^+ buffer solution (100 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 10 mM HEPES, pH 7.4). The oocytes were incubated in 0.1 ml of the Na^+ buffer solution containing either [^{14}C]metformin (20, 100, 500, 1000, 2000, 5000 and 10000 μM) or [^{14}C]phenformin (1, 5, 10, 50, 100, 500 and 1000 μM) at room temperature. After incubation for 90 min, the uptake was terminated by aspiration of the Na^+ buffer solution followed by washing of the oocytes three times with 3 ml of ice-cold Na^+ buffer solution to remove the remaining radiolabeled substrates and to prevent further uptake. Each single oocyte was placed into a vial containing 0.15 ml of 10% (w/v) sodium dodecyl sulfate (SDS) in Na^+ buffer solution and allowed to lyse. This was followed by the addition of 5 ml of liquid scintillator. The radioactivity was determined with a liquid scintillation counter. As a control, the same procedure was operated using oocytes injected with water in place of the oocytes expressing hOCT1.

Data analysis

The concentration dependence of metformin and phenformin transport by hepatocytes and hOCT1 expressing oocytes was analysed using Michaelis-Menten plots based on the following equation:

$$v = V_{max} \times S/(K_m + S) + P_{dif} \times S$$

where v is uptake rate, V_{max} is the maximum uptake rate, S is the concentration of metformin or phenformin, K_m is the Michaelis-Menten constant and P_{dif} is a non-saturable uptake clearance, respectively. The uptake data were fitted to the above equation by nonlinear least-squares regression analysis (MULTI).

Statistical analysis

Data are expressed as mean ± SD. The Student's *t*-test was used to evaluate differences between various treatment groups. A value of $p < 0.05$ was considered to be significant.

Results

Uptake by human hepatocytes

The uptake of [^{14}C]metformin (20 μM) and [^{14}C]phenformin (20 μM) by human hepatocytes at 37°C was higher than that at 0°C. The uptake of metformin increased up to 20 min and that of phenformin increased up to 10 min, respectively (Figure 1). Saturable concentration dependent

DOI: 10.1002/bdd

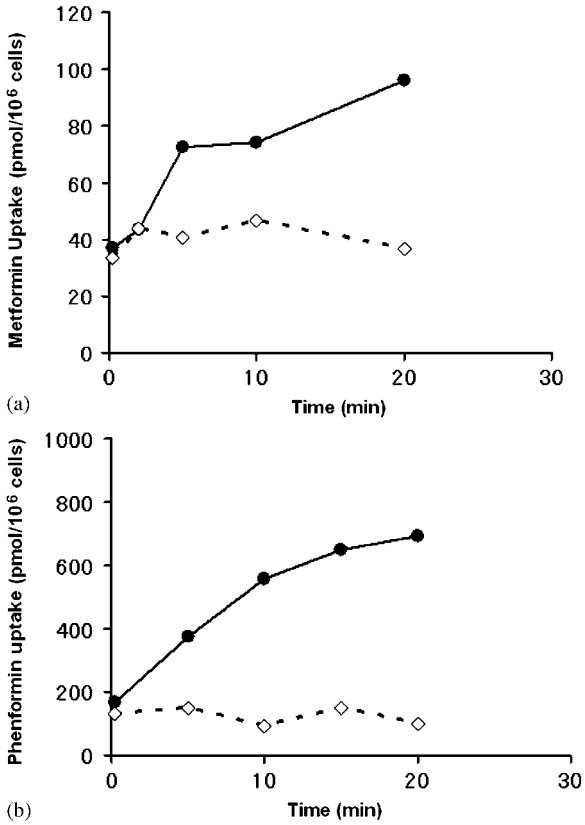

Figure 1. Time course of [^{14}C]metformin (20 μM) (a) and [^{14}C]phenformin (20 μM) (b) uptake by human hepatocytes. Both compounds were incubated for the designated periods at 0°C (empty circle) and 37°C (filled circle). Each point represents the mean of two measurements

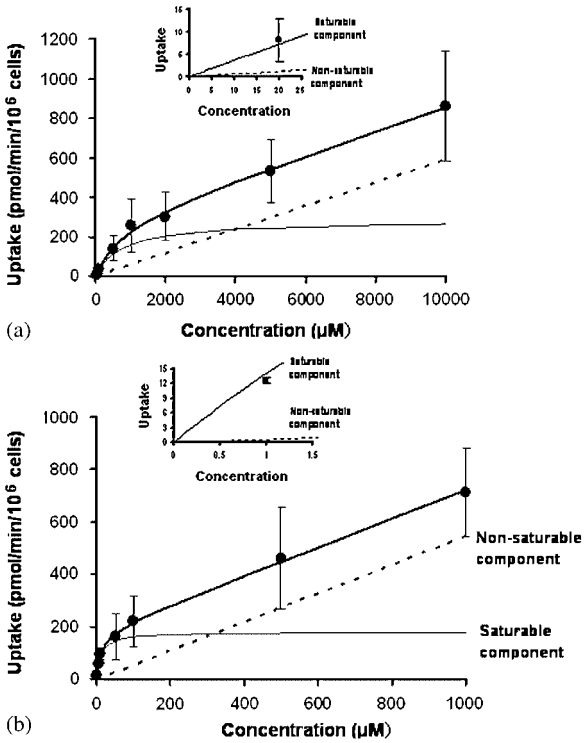

Figure 2. Concentration dependence of uptake of [^{14}C]-metformin (a) and [^{14}C]phenformin (b) by human hepatocytes. [^{14}C]metformin was incubated in a concentration range from 20 to 10000 μM at 37°C for 20 min. [^{14}C]phenformin was incubated in a concentration range from 1 to 1000 μM at 37°C for 10 min. Experiments were conducted using hepatocytes from three different donors. Each point represents the mean ± SD. Insert: the expansion of the clinical concentration area of both biguanides

uptake of both compounds was shown (Figure 2). These data suggest that metformin and phenformin are taken up into human hepatocytes via an active transport mechanism. The Eadie-Hofstee plot (Figure 3) shows that the uptake of [^{14}C]metformin and [^{14}C]phenformin consist of one saturable and non-saturable component. The estimated kinetic parameters are summarized in Table 1. The K_m value of metformin was much higher than that of phenformin. The V_{max} values of metformin and phenformin were not significantly different and the P_{dif} value of metformin was lower than that of phenformin.

Effects of membrane potential on the transport of [^{14}C]metformin and [^{14}C]phenformin

The effect of membrane potential on the uptake of [^{14}C]metformin and [^{14}C]phenformin in human hepatocytes was examined. The accumulation of both compounds in hepatocytes was decreased in the presence of high K^+ (145 mM) medium. The mean percentage [^{14}C]metformin and [^{14}C]phenformin accumulation, with 100% set as the control, was 12.5% and 8.6%, respectively (Figure 4).

Inhibitory effects of TEA and PHA on [^{14}C]metformin and [^{14}C]phenformin uptake.

The inhibitory effects of cationic and anionic compounds on the uptake of [^{14}C]metformin and [^{14}C]phenformin by human hepatocytes were examined. Tetraethylammonium (TEA, a substrate for organic cation transport) and *p*-aminohippuric acid (PHA, a substrate for organic anion transport) were used. TEA showed a potent inhibitory effect,

DOI: 10.1002/bdd

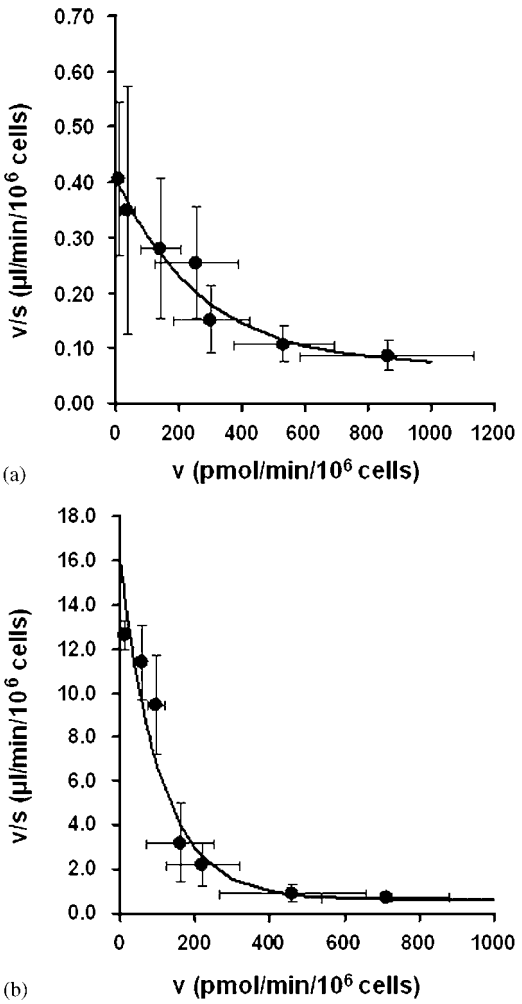

Figure 3. Eadie-Hofstee plots for the uptake of [^{14}C]metformin (a) and [^{14}C]phenformin (b) in human hepatocytes. Each point represents the mean ± SD of three different donors

Table 1. Kinetic parameters for the uptake of [^{14}C]metformin and [^{14}C]phenformin by human hepatocytes

Compound	K_m µM	V_{max} pmol/min/10^6 cells	P_{dif} µl/min/10^6 cells
Metformin	907 ± 324	287 ± 112	0.0588 ± 0.0154
Phenformin	12.5 ± 10.5	192 ± 145	0.527 ± 0.054

The data are mean ± SD with three different batches of hepatocytes.

whereas PHA had no significant influence on the transport of metformin and phenformin into human hepatocytes (Figure 5).

Uptake in oocytes expressing hOCT1

The uptake of both [^{14}C]metformin (20 µM) and [^{14}C]phenformin (20 µM) by hOCT1 expressing oocytes was greater than by water-injected oocytes. The uptake increased up to 90 min (Figure 6) and saturable concentration dependence of both metformin and phenformin uptake was shown in hOCT1 expressing oocytes (Figure 7). The estimated kinetic parameters are summarized in Table 2. The K_m value of metformin was much higher than that of phenformin. The V_{max} values of metformin and phenformin were not so different and the P_{dif} value of metformin was lower than that of phenformin.

Discussion

In the present study, comparison of the uptake characteristics of metformin and phenformin in cryopreserved human hepatocytes and hOCT1 expressing oocytes using ^{14}C-labelled compounds demonstrated major differences between the two drugs. In cryopreserved human hepatocytes, concentration-dependent uptake of both biguanides was observed, however, the K_m value for metformin (907 µM) was much greater than for phenformin (12.5 µM), indicating a lower affinity for transport. The uptake of biguanides was inhibited by the addition of TEA, a typical substrate for OCT. Furthermore, it was suppressed in the presence of high K^+ (118 mM) medium buffer to reduce membrane potential effects. It has been reported that transcellular transport of cationic compounds mediated by OCT is driven by the membrane potential [19]. Therefore, the uptake characteristics in hOCT1 expressing oocytes were examined to characterize the role of transport. Concentration-dependent uptake was again observed for both biguanides, with a K_m value for metformin of 932 µM and for phenformin of 5.6 µM. Values in oocytes were thus comparable to those obtained with human hepatocytes, as well as with previously reported data for human hepatocytes and oocytes [7–9]. These results suggest that metformin and phenformin are transported actively by hOCT1 into hepatocytes. It has been reported that hOCT1 is expressed mainly on the

DOI: 10.1002/bdd

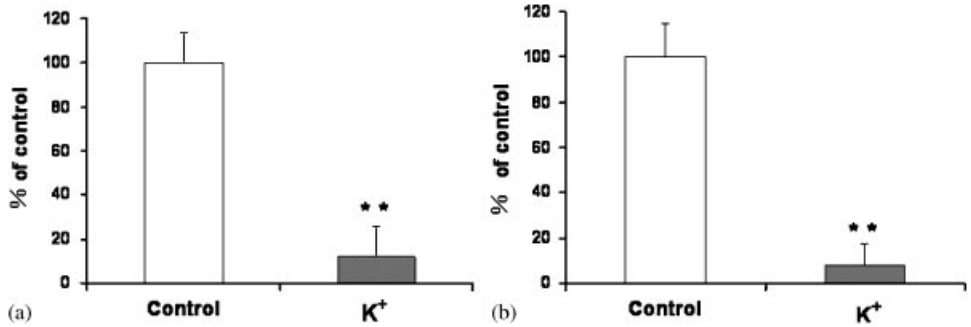

Figure 4. Effect of membrane potential on [^{14}C]metformin (a) and [^{14}C]phenformin (b) uptake by human hepatocytes. The hepatocytes were incubated with high K^+ incubation medium at 37°C for 10 min with 20 μM [^{14}C]metformin (a) or for 5 min with 1 μM [^{14}C]phenformin (b). Each column represents the mean ± SD of three measurements. **Significant difference from the control ($p < 0.01$)

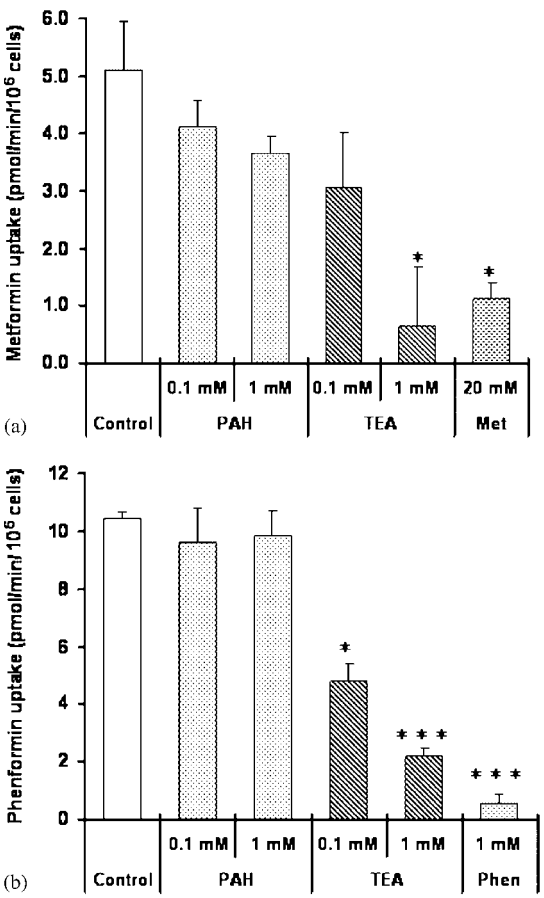

Figure 5. Inhibitory effect of PHA and TEA on [^{14}C]metformin (a) and [^{14}C]phenformin (b) uptake by human hepatocytes. The hepatocytes were incubated with respective compounds at 37°C for 10 min with 20 μM [^{14}C]metformin (a) or for 5 min with 1 μM [^{14}C]phenformin (b). Each column represents the mean ± SD of three measurements. Asterisks indicate a significant difference from the control ($^{*}p < 0.05$, $^{***}p < 0.001$)

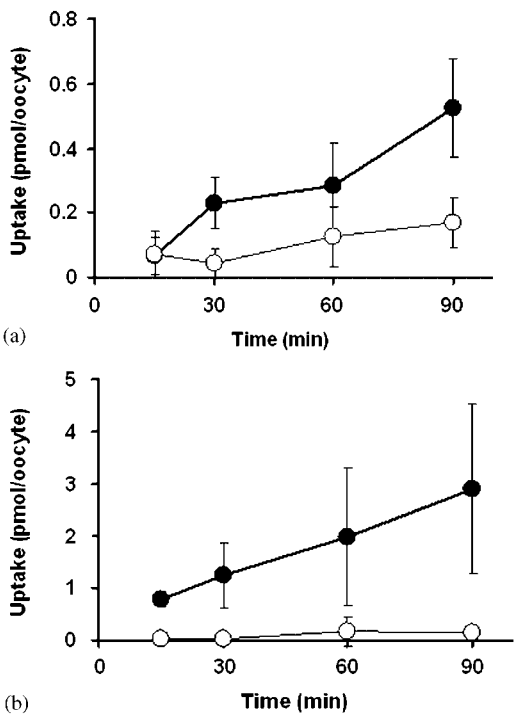

Figure 6. Time course of [^{14}C]metformin (20 μM) (a) and [^{14}C]phenformin (20 μM) (b) uptake by hOCT1 expressing oocytes. Both compounds were incubated for the designated periods in hOCT1 (filled circle) and water injected oocytes as a control (empty circle). Each point represents the mean ± SD of ten oocytes

basolateral membranes of hepatocytes [10,11], the main target organ of biguanides.

It has also been reported that hOCT1 exhibits polymorphic variation [20–23]. Of a total of 15 substitution variants of hOCT1, five exhibited

DOI: 10.1002/bdd

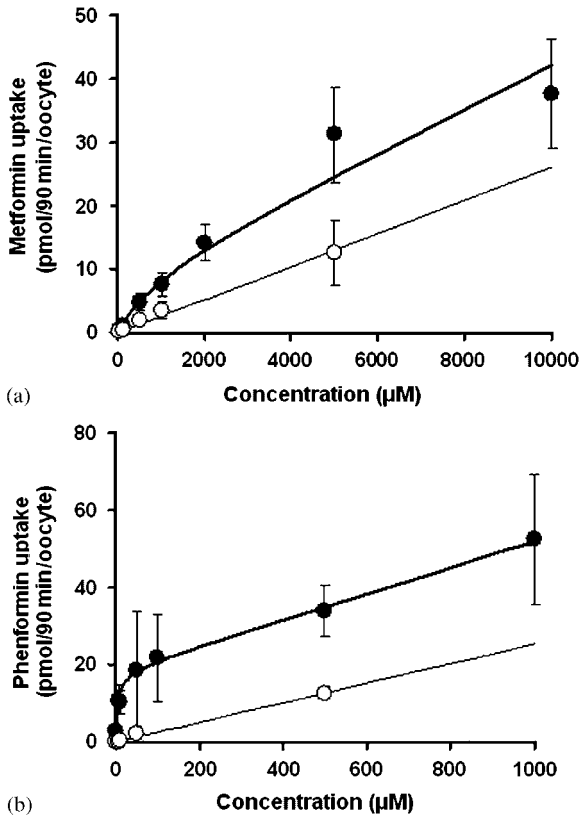

Figure 7. Concentration dependence of uptake of [^{14}C]-metformin (a) and [^{14}C]phenformin (b) by oocytes stably expressing hOCT1 (filled circle) and water injected oocytes as a control (empty circle). [^{14}C]metformin was incubated in a concentration range from 20 to 10000 μM at 37°C for 90 min. [^{14}C]phenformin was incubated in a concentration range from 1 to 1000 μM at 37°C for 90 min. Each point represents the mean ± SD of ten oocytes

Table 2. Kinetic parameters for the uptake of [^{14}C]metformin and [^{14}C]phenformin by hOCT1 expressing oocytes

Compound	K_m μM	V_{max} pmol/min/oocyte	P_{dif} μl/min/oocyte
Metformin	932	8.94	0.00340
Phenformin	5.59	1.83	0.0338

The data are mean values for ten oocytes.

decreased function and one had increased function [21]. Shu *et al.* [12,13] also reported pronounced effects of genetic variation in hOCT1 on metformin pharmacokinetics and pharmacodynamics in a group of healthy volunteers. Wang *et al.* [14] reported that the accumulation of metformin in the liver in Oct1(−/−) mice was more than 30 times lower than in Oct1(+/+) mice, with similar results for blood lactate. Oct1 may thus act as a regulation factor for lactic acidosis. Therefore, if the variability of pharmacokinetic interactions of phenformin with hOCT1 is greater than for metformin, it is likely to show a higher frequency of lactic acidosis. There is a distinct possibility that hOCT1 is a crucial risk factor for PK variability, leading to the difference in frequency of lactic acidosis between metformin and phenformin. The plasma concentrations of metformin and phenformin after oral administration (500 mg/man in metformin, 50 mg/man in phenformin) were 1.55 μg/ml (12 μM) and 99.8–152 ng/ml (0.49–0.74 μM), respectively [24,25] Figure 2 (insert) shows the expansion of the clinical concentration area. At clinical concentrations, the rates of saturable components of metformin and phenformin were about 6-fold and 30-fold greater than those of non-saturable components, respectively. This indicates that both metfomin and phenformin are transported actively by hOCT1 and functional changes in hOCT1 would affect the transport of both biguanides to approximately the same degree. Furthermore, the difference of therapeutic range between metformin and phenformin should be considered. If phenformin has a narrower therapeutic range compared with metformin, it may cause a higher incidence of lactic acidosis. However, it is not clear that there is marked difference in the therapeutic range narrowness between the two biguanides [26–30]. Therefore, it can be speculated that hOCT1 alone does not determine the frequency of drug-induced lactic acidosis. Other factors, such as variation in metabolism, may be related to a variation in the frequency of lactic acidosis between metformin and phenformin in clinical usage. It is believed that phenformin is metabolized extensively *in vivo* by CYP2D6, similar to debrisoquine [31], whereas metformin is not [24]. CYP2D6 exhibits polymorphic variation and about 7% of Caucasians are poor metabolizers (PM) [32]. CYP2D6 could clearly be related to inter-individual variation of drug metabolism. Oates *et al.* [25] demonstrated that the plasma concentration of phenformin was greater in PM than in extensive metabolizers (EM) and the blood lactate concentration increased significantly in PM. These findings suggest that

DOI: 10.1002/bdd

the underlying polymorphism might be related to phenformin-induced lactic acidosis. In fact, blood lactate levels after a single dose of phenformin have been found to be greater in subjects with impaired phenformin metabolism than in others. However, the PM ratio of CYP2D6 (7%) alone cannot fully explain the frequency of lactic acidosis with phenformin (0.04%).

In conclusion, a comparison of the uptake profile between metformin and phenformin was performed for the first time in human cryopreserved hepatocytes and hOCT1 expressing oocytes. Both biguanides are good substrates for hOCT1, however, phenformin has a much greater affinity and is therefore more readily transported. Although there are differences in the uptake kinetics between metformin and phenformin, it can be speculated that hOCT1 alone does not determine the frequency of drug-induced lactic acidosis, based on the therapeutic ranges of both drugs and the involvement of CYP2D6. Other factors, such as the therapeutic situation for patients with renal disease, co-administered drug usage and metabolism, are likely to be involved.

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DOI: 10.1002/bdd