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Blockade of P-Glycoprotein Decreased the Disposition of Phenformin and Increased Plasma Lactate Level

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Abstract

This study aimed to investigate the *in vivo* relevance of P-glycoprotein (P-gp) in the pharmacokinetics and adverse effect of phenformin. To investigate the involvement of P-gp in the transport of phenformin, a bi-directional transport of phenformin was carried out in LLC-PK1 cells overexpressing P-gp, LLC-PK1-Pgp. Basal to apical transport of phenformin was 3.9-fold greater than apical to basal transport and became saturated with increasing phenformin concentration (2-75 μM) in LLC-PK1-Pgp, suggesting the involvement of P-gp in phenformin transport. Intrinsic clearance mediated by P-gp was 1.9 μL/min while passive diffusion clearance was 0.31 μL/min. Thus, P-gp contributed more to phenformin transport than passive diffusion. To investigate the contribution of P-gp on the pharmacokinetics and adverse effect of phenformin, the effects of verapamil, a P-gp inhibitor, on the pharmacokinetics of phenformin were also examined in rats. The plasma concentrations of phenformin were increased following oral administration of phenformin and intravenous verapamil infusion compared with those administerd phenformin alone. Pharmacokinetic parameters such as C_{max} and AUC of phenformin increased and CL/F and Vss/F decreased as a consequence of verapamil treatment. These results suggested that P-gp blockade by verapamil may decrease the phenformin disposition and increase plasma phenformin concentrations. P-gp inhibition by verapamil treatment also increased plasma lactate concentration, which is a crucial adverse event of phenformin. In conclusion, P-gp may play an important role in phenformin transport process and, therefore, contribute to the modulation of pharmacokinetics of phenformin and onset of plasma lactate level.

Key Words: Phenformin, P-gp, Intestinal absorption, Elimination, Plasma lactate concentration

Metformin and phenformin are biguanides that have developed for the treatment of type 2 diabetes. Mechanistically, biguanides increase the activity of adenosine monophosphate-activated protein kinase, a key enzyme that regulates glucose metabolism, lipid metabolism, and energy imbalance (Kim *et al*., 2012; Chang *et al*., 2013). However, biguanides have been associated with lactic acidosis, a potentially fatal adverse reaction. The incidence of lactic acidosis in phenformin users was much higher than that in metformin users, explaining the withdrawal of phenformin from the mark*et al*though metformin has been used as first-line therapeutics in patients with type 2 diabetes (Sogame *et al*., 2013). High plasma concentration of biguanide was reported to be a risk factor for lactic acidosis, a consequence of overproduction of lactate due to inhibition of mitochondrial respiration and increased anaerobic glycolysis (Wang *et al*., 2003; Choi *et al*., 2007).

Hence, investigations on the relevant transport mechanisms of metformin and phenformin have focused on differences in their affinity for drug transporters and resultant plasma concentration to explain the high incidence of lactic acidosis in case of phenformin vs. low incidence of lactic acidosis in metformin. Among the drug transporters investigated, organic cation transporters (OCTs) play a key role in the pharmacokinetics of biguanides (Sirtori *et al*., 1978; Tucker *et al*., 1981). OCT1 mediates hepatic uptake of biguanide drugs, and biguanides are eliminated by the kidney via OCT2-mediated tubular secretion. Sogame *et al*. reported that metformin and phenformin were good substrates for OCT1 and OCT2, but the affinity of phenformin was much higher than that of metformin (Wang *et al*., 2003; Sogame *et al*., 2009; Sogame *et al*., 2011; Sogame *et al*., 2013). Since the plasma lactate level induced by biguanide was correlated with the function of OCT1 (Wang *et al*., 2003), the difference in OCT1-mediated transport may explain the variation in lactic acidosis incidence

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between metformin and phenformin. Moreover, the higher affinity of phenformin for OCT1 could maintain a higher intrahepatic concentration of phenformin, which may inhibit lactate metabolism in the liver and increase incidence risk of lactic acidosis (Wang *et al*., 2003; Sogame *et al*., 2009; Sogame *et al*., 2011).

Besides OCTs transporters, P-glycoprotein (P-gp) have attracted our attention because it is well known that P-gp is distributed in the liver and kidney and play a crucial role in the disposition of organic cationic substrate drugs (Choi and Song, 2012). When comparing the elimination of metformin with that of phenformin, only a small fraction of orally administered metformin is excreted via the biliary route, while a substantial portion of intraduodenally administered phenformin is excreted via this route (26% of dose in 6 h) in rats (Guest *et al*., 1979). Marked biliary excretion of phenformin was observed when the drug was administered at lower doses (37% for 7 mg/kg oral dose vs. 14% for 100 mg/kg oral dose), and the biliary concentrations of the drug were much higher than the blood concentrations at all time-points examined (Guest *et al*., 1979). These observations suggested that biliary excretion of phenformin is mediated by the active efflux process in rats, but details regarding the efflux transport mechanism of phenformin remain unclear.

Therefore, in this study, we evaluated the involvement of P-gp, a major efflux transporter involved in the transport of organic cationic drugs such as anti-cancer drugs, primary and tertiary ammines, and biguanides (Song *et al*., 2013), in the transport mechanism of phenformin and its contribution to the pharmacokinetics of phenformin using *in vitro* LLC-PK1 cells overexpressing P-gp (i.e., LLC-PK1-Pgp cells) and *in vivo* rats. Moreover, we also examined the involvement of P-gp in the alteration of plasma lactate level following the administration of phenformin.

MATERIALS AND METHODS

Materials

Phenformin, verapamil, cyclosporine A (CsA), propranolol, and Hank's Balanced Salts Solution (HBSS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). [^{3}H]digoxin (1.103 TBq/mmol) was purchased from Perkin Elmer Inc. (Boston, MA, USA). Fetal bovine serum (FBS), Medium 199, gentamycin, hygromycin, and trypsin-EDTA were purchased from Hyclone Laboratories. All other chemicals were of reagent grade.

Transepithelial Transport Study

LLC-PK1-Pgp cells (BD-Corning, Corning, NY, USA) were grown in tissue culture flasks in Medium 199 supplemented with 10% FBS, 50 μg/mL gentamycin, and 50 μg/mL hygromycin. The cells were maintained at 37°C in a humidified atmosphere with 5% CO_2/95% air. The cells were grown and seeded on filter inserts of 12 transwell plates at a density of 5×10^5 cells/insert. The integrity of the cell monolayers was evaluated prior to transport experiments by measuring transepithelial electrical resistance, where values in the range of 300-650 $\Omega\cdot cm^2$ were considered appropriate for use in the transport experiment (Choi and Song, 2012).

To investigate whether phenformin is a substrate of P-gp, we measured the apical to basal (A to B) transport and B to A transport of phenformin. For measurement of A to B transport, 0.5 mL of HBSS supplemented with 10 mM HEPES (pH 7.4), 4 mM $NaHCO_3$, and 10 mM glucose containing phenformin (2 μM) in the presence and absence of cyclosporine A (CsA, 25 μM) was added to the apical side, and 1.5 mL of fresh HBSS medium was added to the basal side of the insert. The insert was transferred to a well containing fresh HBSS medium every 15 min for 1 h. For measurement of B to A transport, 1.5 mL of HBSS medium containing phenformin (2 μM) in the presence and absence of CsA (25 μM) was added to the basal side, and 0.5 mL of fresh HBSS medium was added to the apical side. The transport medium on the apical side was replaced with 0.4 mL of fresh incubation medium every 15 min for 1 h.

Concentration dependency in the B to A transport of phenformin was measured in concentrations of 2, 5, 10, 35, 50, 75 μM and the procedure was identical to the procedure above mentioned. A 100 μL aliquot of each sample was added to a 100 μL aliquot of acetonitrile containing 1 ng/mL of propranolol. After vortex mixing for 10 min and centrifugation for 10 min at 13,000 rpm, the supernatant was transferred to a clean tube and evaporated under a gentle stream of nitrogen gas. The residue was reconstituted in 150 μL of mobile phase, and a 2 μL aliquot was injected directly into the liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) system.

To investigate the effect of phenformin on P-gp transport activity, we measured the B to A transport of [^{3}H]digoxin in LLC-PK1-P-gp cell monolayers. Aliquots (1.5 mL) of HBSS medium containing 0.1 μM [^{3}H]digoxin and phenformin (0, 1, 2.5, 5, 10, and 30 μM) were added to the basal side, and 0.5 mL of fresh HBSS medium was added to the apical side. The transport medium in the apical side was replaced with 0.4 mL of fresh incubation medium every 15 min for 1 h. Aliquots (100 μL) of samples were mixed with scintillation cocktail, and the radioactivity of the probe substrate in the medium was measured using a liquid scintillation counter, MicroBeta (Perkin Elmer). The A to B transport rate of 0.1 μM [^{3}H]digoxin in the presence of phenformin (0, 1, 2.5, 5, 10, and 30 μM) was measured similar to the method described above.

Pharmacokinetic study

Male Sprague-Dawley (SD) rats (7-8 weeks, 220-250 g) were purchased from the Samtako Co. (Osan, Korea). Animals were acclimatized for 1 week in a temperature controlled room (23 ± 2ºC), with a relative humidity of 55 ± 10%, an illumination intensity of 150-300 lux, a frequency of air ventilation of 15-20 times/h, and a 12 h illumination cycle (07:00-19:00). Food and water were supplied *ad libitum*. All animal procedures were approved by the Animal Care and Use Committee of Kyungpook National University.

The femoral artery and vein were cannulated with polyethylene tubes (PE-50; Jungdo, Seoul, Korea) under light anesthesia with isoflurane. The rats were not restrained at any time during the study. Heparinized isotonic saline (10 U/mL) was used to flush the catheters to prevent blood clotting. The rats fasted for at least 12 h before the oral administration of drugs.

Phenformin was dissolved in water, and a 2 mg/kg dose was injected via the femoral vein (vehicle dosing volume, 1 mL/kg). Blood samples (approximately 250 μL each) were collected from the femoral artery at 0, 0.05, 0.083, 0.17, 0.25, 0.5, 1, 2, 3, 4, and 6 h following intravenous administration. Phenformin was also administered to the rats by oral gavage (vehicle dosing volume, 3 mL/kg) at 100 mg/kg. Blood samples (approximately 250 μL each) were collected from the femoral

artery at 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, and 10 h following oral administration of phenformin. After centrifugation of blood samples at 13,000 rpm for 5 min, plasma samples (100 μL) were collected and stored at -80ºC until analysis.

To investigate the effect of P-gp inhibition on the pharmacokinetics of phenformin, phenformin was administered to rats in the presence and absence of verapamil, a P-gp inhibitor. Verapamil was infused to rats at a dose of 2.5 mg/kg/h after a single intravenous administration of 1.5 mg/kg dose. Thirty min after starting verapamil infusion, phenformin was administered orally at a dose of 100 mg/kg. Subsequent procedures were performed as described above.

Aliquots of 50 μL plasma were added to 200 μL of acetonitrile containing 0.5 ng/mL of propranolol (an internal standard). After vortex mixing for 10 min and centrifugation for 10 min at 13,000 rpm, the supernatant was transferred to a clean tube and evaporated under a gentle stream of nitrogen gas. The residue was reconstituted in 150 μL of mobile phase, and a 5 μL aliquot was injected directly into LC-MS/MS system.

LC-MS/MS analysis of phenformin and verapamil

Agilent 6430 Triple Quad LC-MS/MS system (Agilent, Wilmington, DE, USA) coupled with an Agilent 1290 series high performance liquid chromatography system was used. Separation was performed on a Luna CN (2.0 mm i.d.×150 mm, 5 μm, Phenomenex, Torrence, CA, USA) using a mobile phase that consisted of methanol and water (70:30, v/v) containing 0.1% formic acid at a flow rate of 0.2 mL/min. Retention time was 2.8 min for phenformin, 4.9 min for verapamil, and 3.8 min for propranolol (IS). Mass spectra were recorded by electrospray ionization in a positive mode. Quantification was carried out using selected reaction monitoring at m/z 206 → 60 for phenformin, m/z 455.3 → 165.3 for verapamil, and m/z 260.0 → 116.0 for propranolol. Calibration was applied on a standard curve in the range of 2-3000 nM for both compounds. The lower limit of quantitation of both phenformin and verapamil was defined as 2 nM. Intra-day/inter-day precision and accuracy were found to be within the acceptance criteria for assay validation.

Measurement of lactic acid

Plasma lactic acid levels were determined by an enzymatic colorimetric assay using a lactate assay kit (Sigma-Aldrich). Briefly, 50 μL aliquot of plasma samples withdrawn from rats 10 h after the administration of phenformin (per oral, 100 mg/kg) in the presence and absence of verapamil infusion (2.5 mg/kg/h for 10 h) with loading injection (1.5 mg/kg) were directly added in triplicate to a 96-well plate and mixed with 50 μL of master reaction buffer containing lactate enzyme and probe. The mixture was incubated for 30 min at room temperature under dark conditions. The absorbance of the prepared solution was measured directly using an ultraviolet (UV) spectrophotometer (Shimadzu UV-1800) at a wavelength of 570 nm.

Data analysis

In each transport experiment, the apparent initial transport rate (V, pmol/cm^2/min) of the drug was calculated from the initial linear portion of the plot of the total amount of drug transported versus time and measured for various initial drug concentrations in the donor compartment of the insert. Efflux ratio (ER) was calculated from the mean ratio of B to A transport rate to A to B transport rate of substrate, such as phenformin and digoxin. An ER value greater than 2 represented P-gp-mediated efflux transport.

By using a concentration-dependent B to A transport of phenformin, a non-linear regression analysis was performed to fit the plot of the following modified Michaelis-Menten equation:

$$V = V_{max} \times \frac{S}{K_m + S} + K_d \times S. \text{ Where, } V_{max} \text{ and } K_m$$

Where, V_{max} and K_m are the maximum transport rate (pmol/cm^2/min) and the Michaelis-Menten constant (μM), respectively, and Kd represents the diffusion clearance (μL/cm^2/min). The intrinsic clearance for the transport (CL_{int}) was obtained from V_{max}/K_m.

In the inhibition studies, ER was calculated according to the ratio of B to A transport rate to A to B transport rate of digoxin in the presence and absence of phenformin. Relevant data were fitted to an inhibitory effect model (Zhang *et al*., 2006).

$$[\text{i.e., } v = E_{max}(1 - \frac{[I]}{IC_{50} + [I]})]$$

IC_{50} value reflects the concentration of inhibitor to show half-maximal inhibition.

In the *in vivo* rat studies, non-compartmental pharmacokinetic analysis was performed using the WinNonlin (ver. 2.0, Pharsights, Mountain View, CA, USA). The area under the plasma concentration-time curve (AUC_∞) was the sum of AUC from zero to last sampling time (AUC_{last}) calculated using the linear trapezoidal method and AUC from last sampling time to infinity ($AUC_{last-\infty}$) estimated by dividing the last measured concentration in plasma by the terminal rate constant. The terminal elimination half-life ($T_{1/2}$) was calculated by dividing 0.693 with the slope of the terminal phase. Systemic clearance (CL) was determined by dividing dose by AUC.

Statistical comparisons were performed by t-test using Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL, USA), where $p<0.05$ was considered statistically significant.

RESULTS

Transport rate of phenformin in LLC-PK1-Pgp cell monolayer

The B to A transport rate of phenformin in LLC-PK1-Pgp was about 13.2-fold greater than A to B transport. The presence of CsA decreased the B to A transport rate of phenformin by 86% without changing the A to B transport rate, resulting in decreased ER from 13.1 to 1.9 (Fig. 1). These results indicated the involvement of P-gp in the efflux of phenformin in LLC-PK1-Pgp cells. To characterize the kinetic parameters of P-gp-mediated efflux of phenformin, we measured concentration-dependent B to A transport of phenformin in the presence of this drug (2-75 μM). The transport rate became saturated with increasing phenformin concentration. The data fit the equation of Michaelis-Menten and passive diffusion, yielding the kinetic parameters: V_{max} of 8.5 pmol/min/cm^2, K_m of 4.6 μM, C_{Lint} of 1.9 μL/min/cm^2, and diffusion coefficient K_d of 0.31 μL/min/cm^2 (Fig. 2).

We further investigated the involvement of P-gp in the transport of phenformin, examining the effect of phenformin

on the transport rate of digoxin in LLC-PK1-Pgp cells. The B to A transport rate of digoxin was significantly inhibited by phenformin in a concentration-dependent manner, whereas A to B transport of digoxin was not significantly affected (Fig. 3A). Consequently, the ER of digoxin decreased in the presence of phenformin in a concentration-dependent manner, yielding an IC_{50} value of 1.8 μM (Fig. 3B). These results suggested the involvement of P-gp in the transport of phenformin with a high affinity.

Pharmacokinetics of phenformin

Bioavailability of phenformin administered as a single oral dose of 100 mg/kg, which is comparable to the therapeutic dose in humans (Bando *et al*., 2010), was assessed by comparing with the plasma profiles of this drug following intravenous administration of phenformin. Plasma concentration profile of intravenously administered phenformin exhibited a multi-exponential elimination process (Fig. 4A) and the pharmacokinetic parameters of phenformin are shown in Table 1. The plasma concentration profile of orally administered phenformin is shown in Fig. 4B and the pharmacokinetic parameters are shown in Table 1. Oral bioavailability of phenformin was calculated as 39 ± 3%.

To investigate the effect of P-gp inhibition on the pharmacokinetics of phenformin, verapamil, a representative P-gp inhibitor (Bansal *et al*., 2009), was infused to rats at a dose of 2.5 mg/kg/h for 10 h with a loading dose of 1.5 mg/kg. Plasma concentrations of verapamil reached a steady state after 2 h and were maintained at 2.8 ± 0.3 μM (Fig. 4B). Verapamil increased C_{max} and AUC of phenformin but decreased CL/F and V_{ss}/F. However, $t_{1/2}$ remained unaltered after verapamil treatment (Table 2). These data suggested that increasing plasma concentration of phenformin increased C_{max} and AUC values. This was mainly due to increased absorption of phenformin, because the plasma concentration of phenformin profoundly increased at early time points but the elimination half-life was not significantly altered (Fig 4C). CL/F and V_{ss}/F of phenformin also decreased after verapamil infusion, which most likely reflected increased plasma concentration of phenformin observed in response to P-gp inhibition (Table 2).

Next, we measured and compared the lactic acid levels in

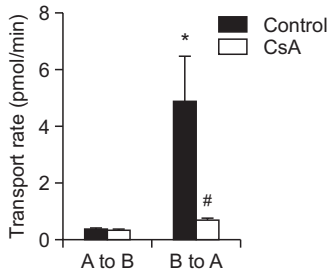

Fig. 1. The apical to basal (A to B) and basal to apical (B to A) transport of 10 μM phenformin across LLC-PK1-Pgp cell monolayers in the presence (gray bar) and absence (black bar) of 25 μM cyclosporine A (CsA). Each data point represents the mean ± SD of three independent experiments. *Statistically different by t-test (p<0.05), compared with the A to B transport rate. #Statistically different by t-test (p<0.05), compared with the B to A transport rate in the absence of CsA.

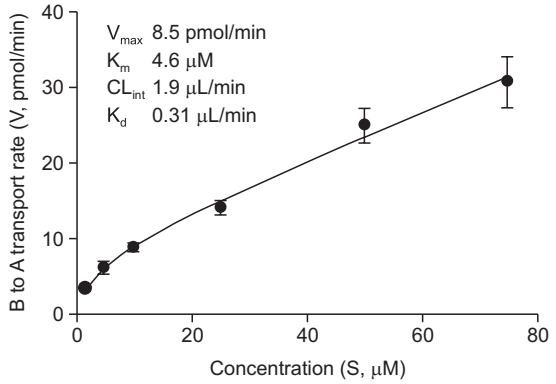

Fig. 2. Concentration dependence of the B to A transport of phenformin (2-75 μM) across LLC-PK1-Pgp cell monolayers. Each data point represents the mean ± SD. Lines were generated from kinetic parameters estimated using a modified Michaelis-Menten equation.

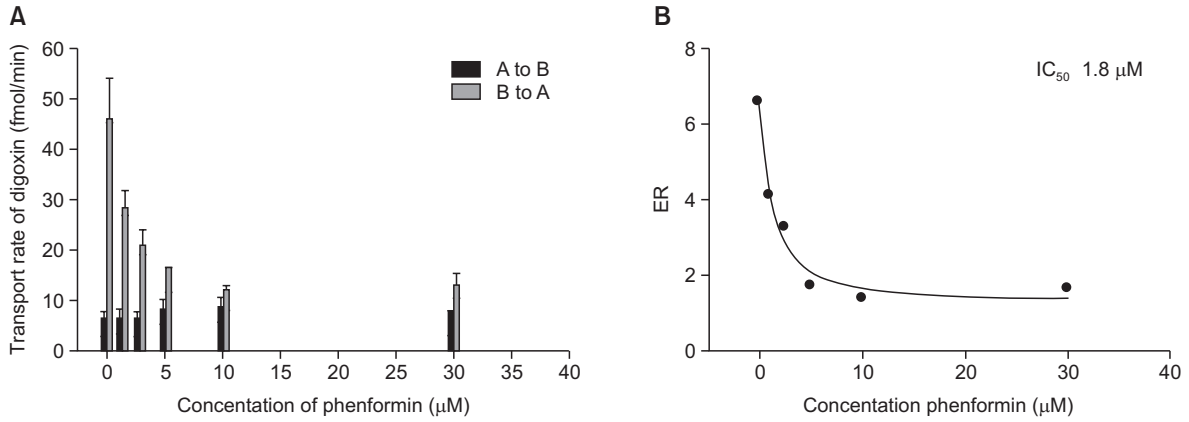

Fig. 3. (A) Inhibitory effect of phenformin (0-30 μM) on the A to B and B to A transport of 0.1 μM [^{3}H]digoxin in LLC-PK1-Pgp cell monolayers. The black bar represents the mean ± SD of three independent experiments. (B) Efflux ratio (ER) of digoxin was shown as a function of phenformin concentration. ER represents the mean ratios of B to A transport rate versus A to B transport rate of digoxin. Data were fitted to an inhibitory effect E_{max}-model and the IC_{50} value was calculated.

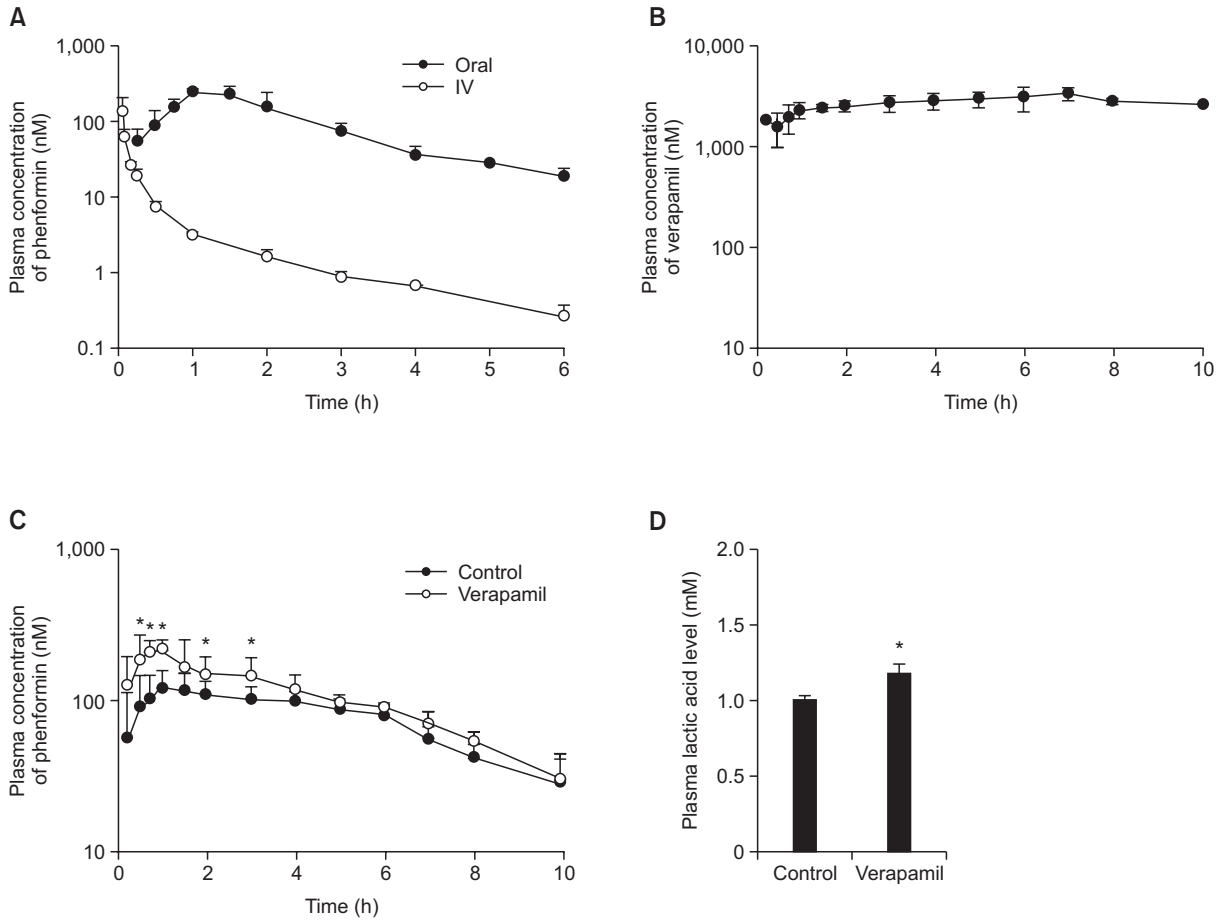

Fig. 4. (A) Plasma concentration profile of phenformin in rats after intravenous (2 mg/kg, ○) and oral (100 mg/kg, ●) administration of phenformin. (B) Plasma concentration profile of verapamil in rats after intravenous bolus injection (1.5 mg/kg) and intravenous infusion (2.5 mg/kg/h) of verapamil for 10 h. (C) Plasma concentration profile of phenformin in rats administered phenformin orally (100 mg/kg) with (○) or without (●) intravenous bolus injection (1.5 mg/kg) and intravenous infusion (2.5 mg/kg/h) of verapamil for 10 h. (D) Plasma concentration of lactic acid 10 hours after oral administration of phenformin (100 mg/kg) with or without intravenous bolus injection (1.5 mg/kg) and intravenous infusion (2.5 mg/kg/h) of verapamil for 10 h. Each data point and bar represents the mean ± SD of five rats. *Statistically different by t-test (p<0.05), compared with the control group.

Table 1. Pharmacokinetic parameters of phenformin after intravenous (IV, 2 mg/kg) and per oral administration (PO, 100 mg/kg) of phenformin

	IV (2 mg/kg)	PO (100 mg/kg)
C_0 (nM)	299.6 ± 90.9	
C_{max} (nM)		163.5 ± 19.4
T_{max} (h)		1.33 ± 0.29
AUC_{last} (nM×h)	29.48 ± 9.76	557.2 ± 31.4
AUC_{∞} (nM×h)	30.47 ± 9.20	600.1 ± 48.4
$T_{1/2}$ (h)	1.68 ± 0.57	2.11 ± 0.68
CL (mL/h/kg)	338.1 ± 89.2	
V_{ss} (mL/kg)	194.1 ± 98.1	
BA (%)		39 ± 3

C_0: Plasma concentration at time zero, C_{max}: Maximum plasma concentration, T_{max}: Time that shows maximum plasma concentration, AUC_{last}: The area under the plasma concentration-time curve from zero to last sampling time, AUC_{∞}: The area under the plasma concentration-time curve from zero to infinity, $T_{1/2}$: The terminal elimination half-life, CL: Systemic clearance, V_{ss}: Volume of distribution at steady-state, BA: Bioavailability.

Table 2. Pharmacokinetic parameters of phenformin administered orally at a dose of 100 mg/kg after intravenous bolus injection (1.5 mg/kg) and intravenous infusion of verapamil (2.5 mg/kg/h) for 10 h

	Control	Verapamil
C_{max} (nM)	150.4 ± 4.36	244.0 ± 44.4*
T_{max} (h)	1.67 ± 0.58	1.00 ± 0.41
AUC_{last} (nM×h)	562.3 ± 23.4	858.4 ± 210*
AUC_{∞} (nM×h)	614.8 ± 42.9	973.5 ± 226*
$T_{1/2}$ (h)	2.38 ± 0.96	3.22 ± 1.27
CL/F (mL/h/kg)	795 ± 55	520 ± 114*
V_{ss}/F (mL/kg)	3110 ± 273	1831 ± 488*
BA (%)	40 ± 3	64 ± 15

*Statistically different by t-test (p<0.05), compared with the control group, C_{max}: Maximum plasma concentration, T_{max}: Time that shows maximum plasma concentration, AUC_{last}: The area under the plasma concentration-time curve from zero to last sampling time, AUC_{∞}: The area under the plasma concentration-time curve from zero to infinity, $T_{1/2}$: The terminal elimination half-life, CL/F: Systemic clearance of orally administered drug, V_{ss}: Volume of distribution at steady-state of orally administered drug, BA: Bioavailability.

plasma samples from rats administered phenformin (single oral dose, 100 mg/kg) with and without verapamil (2.5 mg/kg/hr infusion for 10 h and 1.5 mg/kg loading injection). As shown in Fig. 4D, plasma lactic acid level significantly increased in rats co-administered phenformin and verapamil relative to the levels in rats administered phenformin alone. These results suggested that the increased plasma concentration of phenformin caused by the blockage of P-gp could increase the plasma lactic acid production, which may explain the increased risk of lactic acidosis with phenformin.

DISCUSSION

Phenformin, one of the biguanides, was withdrawn from the market in 1977 owing to increased incidence of lactic acidosis, a life-threatening adverse reaction. The potential risk for phenformin-associated lactic acidosis increased in a dose-dependent manner by using a rat dose range comparable to the therapeutic dose range in humans. In contrast, the incidence of lactic acidosis with metformin was 10- to 20-fold lower than that with phenformin (Bando *et al*., 2010). One in 4,000 patients taking phenformin develops lactic acidosis, compared with one in 80,000 in patients taking metformin (Kreisberg and Wood, 1983; Kwong and Brubacher, 1998). In a review of 330 cases of biguanide associated lactic acidosis, 285 cases involved phenformin, and 50.3% of the patients died (Oliva, 1969; Luft *et al*., 1978; Kwong and Brubacher, 1998).

To determine the potential contributors that control the plasma concentration of biguanides and lactic acid production, we have focused on drug transporters, since biguanides are neither metabolized nor bound to plasma protein (Sogame *et al*., 2009; Sogame *et al*., 2011; Sogame *et al*., 2013). Here, we firstly proved the involvement of P-gp in the disposition of phenformin. We found that phenformin is a substrate for P-gp, with K_m value of 4.6 μM, and that P-gp-mediated transport activity was 6.1-fold greater than passive diffusion of phenformin (Fig. 2). These results suggested that P-gp-mediated phenformin efflux might play a crucial role in the pharmacokinetic properties of phenformin. High affinity of phenformin to P-gp was confirmed by the inhibitory effect of phenformin on P-pg-mediated digoxin efflux (Fig. 3).

When we investigated the pharmacokinetic properties of phenformin, we found that the oral bioavailability of phenformin was about 40% (Fig. 4A, Table 1). In contrast, metformin is known to be freely absorbed (about 74%-90% of dose) through the intestine, and is neither a substrate nor an inducer/inhibitor of P-gp (Song *et al*., 2006). Low bioavailability and intestinal absorption of phenformin compared with metformin may be attributed to the role of P-gp in the process of intestinal absorption of this drug. To further investigate the relevance of P-gp in the pharmacokinetic properties of phenformin, we measured alterations in the pharmacokinetics of phenformin after inhibition of P-gp with verapamil (Bansal *et al*., 2009). The IC_{50} of verapamil is about 2-10 μM (Huang and Liu, 1999; Wang *et al*., 2011; Bai *et al*., 2013). To maintain the plasma concentration of verapamil above the IC_{50} value and to avoid cardiac toxicity (Wang *et al*., 2011; Bai *et al*., 2013), we infused verapamil at a rate of 2.5 mg/kg for 10 h. Verapamil co-treatment with phenformin increased phenformin plasma concentration and decreased its clearance (Fig. 4, Table 2).

This verapamil infusion enhanced lactate concentration probably through the inhibition of P-gp, which is consistent with a study by Wang *et al*. (Wang *et al*., 2003). The higher affinity of phenformin for OCT1 could maintain a higher intrahepatic concentration of phenformin, which may inhibit lactate metabolism in the liver and increase incidence risk of lactic acidosis (Wang *et al*., 2003; Sogame *et al*., 2009; Sogame *et al*., 2011). In a similar manner, P-gp can modulate the intestinal absorption and elimination of phenformin, but not metformin (Song *et al*., 2006), and therefore, P-gp inhibition would increase phenformin plasma concentration and lactate level.

In conclusion, the involvement of P-gp in the transport of phenformin with high affinity may restrict its intestinal absorption and facilitate the elimination of phenformin from the body. However, under certain circumstances, P-gp inhibition by drug-drug interaction or disease state would increase phenformin plasma concentration and lactate level and thereby increase the risk of lactic acidosis. Therefore, P-gp can contribute to the pharmacokinetics and adverse effect of phenformin.

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

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