Role of intestinal microbiota in metabolism of voglibose *in vitro* and *in vivo*

Mahesh Raj Nepal, Mi Jeong Kang, Geon Ho Kim, Dong Ho Cha, Jin Sung Kim, Ju-Hyun Kim and Tae Cheon Jeong[#]

College of Pharmacy, Yeungnam University, 280 Daehak-Ro, Gyeongsan 38541,

South Korea

[#]Corresponding author:

Tae Cheon Jeong, Ph.D.

Professor

College of Pharmacy

Yeungnam University

280 Daehak-ro

Gyeongsan, 38541

South Korea

Tel: +82-53-810-2819

Fax: +82-53-810-4654

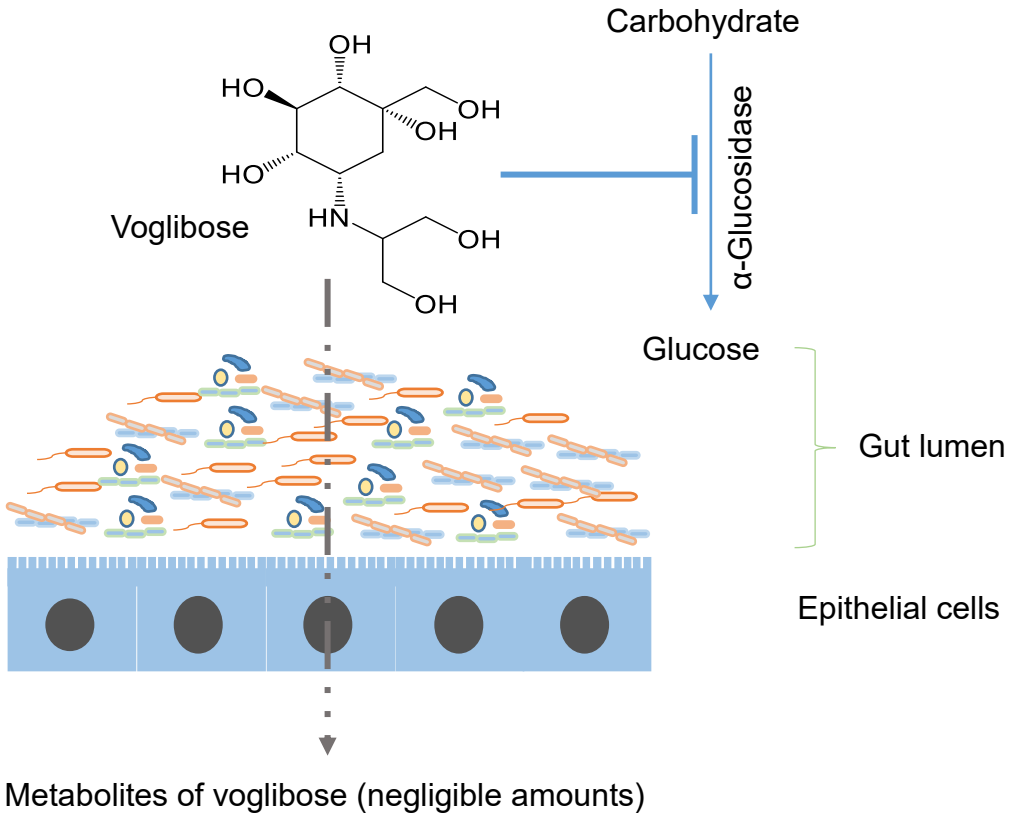
E-mail: taecheon@ynu.ac.kr

Abstract

Voglibose, an α-glucosidase inhibitor, inhibits breakdown of complex carbohydrates into simple sugar units in intestine. Studies proved that none or very less metabolism of voglibose occurred in liver due to its poor intestinal absorption. Trillions of microorganisms reside in intestine and have several roles in metabolism and detoxification of a variety of xenobiotics. Due to the limited information on the metabolism in intestine, the possible metabolism of voglibose by intestinal microbiota was investigated *in vitro* and *in vivo*. For the *in vitro* study, different concentrations of voglibose were incubated with intestinal contents prepared from both vehicle- and antibiotics-treated mice to determine the disappearance of voglibose over the time by using LC-MS/MS. The results indicated that the remained voglibose could be significantly more reduced when incubated with the intestinal contents from normal mice than with those from antibiotics-treated mice that had less enzyme activities. Similarly, *in vivo* pharmacodynamic effect of voglibose was determined following the administration of voglibose and starch in vehicle- and antibiotic-pretreated non-diabetic and diabetic mice, by measuring the modulatory effects of voglibose on blood glucose levels. The results showed that the antibiotic pretreatment to mice resulting in the reduced metabolism of voglibose caused significantly lowered blood glucose levels than control animals. Taken together, the present results clearly indicated that voglibose could be metabolized by intestinal microbiota, and that the metabolism of voglibose by intestinal microbiota would be pharmacodynamically critical in lowering blood glucose level.

Keywords: gut microbiota; voglibose; antibiotics; blood glucose; metabolism

Graphical abstract



1. Introduction

Gut microbiota has been known as a hidden organ of human body for a long time [1]. It is estimated that about 99% of the intestinal microbe population would be of anaerobic types and cover more than 1000 species of bacteria in human and animals [2,3]. In addition, the composition of intestinal bacteria can be affected by age, sex, diet, exercise, and environmental conditions that we live on [4]. Since few decades, researchers have extensively focused to investigate the possible roles of intestinal microbiota in human health and disease, from which it has been found, at least in part, that numerous biological functions including emotion, immunity, and drug metabolism were closely connected with gut microbiota [5]. Among all, drug metabolism by intestinal microbiota might be considered as an important aspect that modulates pharmacodynamic actions of certain drugs [6]. Through a variety of reactions including hydrolysis, intestinal microbiota can play a significant role in the metabolism of drugs administered orally, so that either efficacy or toxicity can be largely modulated [4].

Orally administered drugs would not only be directly absorbed via gastrointestinal tract into liver where extensive metabolism occurs, but also interact with numerous bacteria present in the gut, by which additional metabolism occurs [7,8]. In addition, enterohepatic recycling of xenobiotics would occur by certain enzymes like β-glucuronidase and sulfatase produced by intestinal bacteria [9,10]. By the interaction with intestinal microbiota, properties and fate of certain xenobiotics can be largely altered. Most importantly, the formation of new metabolite(s) might lead to changes in pharmacological or toxicological properties of xenobiotics [11,12]. In fact, the impacts of gut microbiota on xenobiotic metabolism in several animal models have been investigated elsewhere [10,13,14].

Voglibose is an α-glucosidase inhibitor developed in 1994 in Japan for lowering post-prandial blood glucose level in people with type-2 diabetes mellitus [15,16]. It has been observed that voglibose has a minor systemic absorption and negligible metabolism in liver [15]. From our preliminary experiments with several anti-diabetic drugs, it was found that voglibose was significantly disappeared when it was incubated with intestinal content preparations from mice. Therefore, in the present study, we studied, for the first time, the metabolism of voglibose by intestinal microbiota *in vitro* and *in vivo*. For *in vitro* metabolism study, voglibose was incubated with intestinal contents prepared from vehicle- and antibiotics-treated mice using LC-MS/MS. In addition, for *in vivo* study, pharmacodynamic effects of voglibose was determined by administering voglibose followed by starch in antibiotics-treated and -untreated diabetic and non-diabetic mice to determine blood glucose levels, because the absorption of voglibose in intestine would be reportedly limited.

2. Materials and methods

2.1. Materials

Voglibose (> 98.0%) was purchased from Tokyo Chemical Industries (Tokyo, Japan). Erythromycin, oxytetracycline hydrochloride, cefadroxil, 4-nitrophenyl β-D-glucopyranoside, 4-nitrophenyl β-D-glucuronide, 4-nitrophenyl sulfate, soluble starch, ammonium acetate, streptozotocin, and telmisartan, internal standard (IS), were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade acetonitrile was purchased from J. T. Bakers (Central Valley, PA, USA). Anaerobe gas generating system was purchased from Becton, Dickinson and Company (Sparks, MD, USA). Blood glucose monitoring system was purchased from Bayer Contour^{TM} TS (Mishawaka, IN, USA). Potassium phosphate monobasic and potassium

phosphate dibasic were purchased from Duksan (Seoul, South Korea). All other chemicals were of analytical grades and used as received.

2.2. Animals

Male ICR mice of 7 weeks of age were purchased from Orient Bio (Seoul, South Korea). Animals upon received were randomly placed five in a cage. After acclimatization of mice for at least 1 week under controlled temperature of 22 ± 2°C, relative humidity of 50 ± 10%, and air change of 10-20 cycles per hr with light and dark cycle of 12-hr, animals were made ready for the study. The study was performed following procedures of Institutional Animal Care and Use Committee of Yeungnam University (approved No., 2014-008). All animals were allowed to access food and water *ad libitum*.

2.3. Preparation and extraction of intestinal contents for in vitro study

For *in vitro* studies, mice were classified as antibiotics-treated and vehicle-treated groups (n = 5). In antibiotics-treated group, mice were orally administered with a mixture of 3 antibiotics in saline, such as erythromycin (300 mg/kg), oxytetracycline HCl (300 mg/kg) and cefadroxil (100 mg/kg), for three successive days. Saline was administered in vehicle-treated group for three days. Twenty-four hr after the last dose of either antibiotics or vehicle, intestinal contents from both groups were harvested. Collected intestinal contents were mixed with two volumes of potassium phosphate buffer, pH 7.4, vortexed, and homogenized at 11,000 rpm for 2 min. Then, the sample was centrifuged at 500 g for 10 min at 4°C, and the supernatant was collected for *in vitro* tests. Aliquots were prepared and stored at -70°C until their use.

2.4. Determination of intestinal microbial enzyme activity

The enzyme assays were accomplished according to the previously published report with some modifications [17]. Briefly, 0.4 ml of either 2.5 mM 4-nitrophenyl β-D-glucopyranoside, 4-nitrophenyl β-D-glucuronide, or 4-nitrophenyl sulfate in potassium phosphate buffer, pH 7.4, was mixed with 0.4 ml of potassium phosphate buffer, pH 7.4, and 0.2 ml of intestinal contents obtained from both antibiotics- and vehicle-treated mice, followed by the incubation at 37°C for 30 min with shaking. The reaction was terminated by adding 0.25 ml of 1 N NaOH. Following the centrifugation at 3,000 g for 10 min, the absorbance was measured at 405 nm by using a UV spectrophotometer (Eppendorf, Germany). Standard calibration curves were prepared for the calculation of enzyme activities.

2.5. Analytical conditions

An HPLC connected with mass spectrometer (AB Sciex, API-4000) was employed in the present study. The mobile phase was consisted of 10 mM ammonium acetate buffer (mobile phase A) and acetonitrile (mobile phase B). A gradient condition was employed for the quantitative analysis in which the mobile phase B was started from 0.5% and hold for 1 min and increased to 10% in next 2.0 min. And then, mobile phase B was increased to 90% in another 1 min, hold at 90% for 1.5 min, and returned to 1% in another 1.5 min. Column equilibration time was 7.0 min and the total run time was 14.0 min. Analyte peaks were identified with Agilent Eclipse plus C_8 column (2.1 × 150 mm, 3.5 µm). The column temperature was maintained at 30°C with the flow rate of 0.25 ml/min, and the injection volume was 5.0 µl. Multiple reaction monitoring mode (MRM) was used for the quantitative detection of voglibose using the ratio of areas of analyte to IS peaks. Voglibose and telmisartan (IS) was detected in positive ion modes and mass transitions for voglibose and telmisartan were *m/z* 268 → 92 and *m/z* 515 → 276, respectively. The DP, CE and CXP values were 70, 30 and 18 for voglibose and 156, 65 and 20 for telmisartan, respectively.

2.6. Analytical validation

Inter-day and intra-day validations were performed according to the FDA guideline [18]. Accuracy and coefficient of variation (CV %) was calculated based on the analysis of spiked QC samples of different concentrations. QC samples of 4, 100 and 400 ng/ml voglibose were analyzed. For intra-day validation, five freshly prepared samples of each concentration were analyzed in a single day and, for inter-day validation, QC samples were freshly prepared each day and analyzed for five consecutive days. Accuracy and precision of analyzed QC samples were determined.

2.7. Stability studies

To investigate the possible metabolism of voglibose by intestinal microbiota, the remaining level of voglibose was measured following an incubation of voglibose with the intestinal contents and compared with the initial level of voglibose. Prior to this, stability of voglibose was tested with a target CV (%) of 15. Voglibose in potassium phosphate buffer, pH 7.4, at 5, 10 and 20 µg/ml was incubated at various conditions for long term and short-term stability studies. For short-term stability test, voglibose was incubated in both 25°C and 37°C for 24 hr. Similarly, voglibose was tested for long-term stability after storage in a freezer for 30 days at -20°C and 5 days at + 4°C. The stability of voglibose was also tested following three freeze-thaw cycles from –20°C to room temperature.

2.8. Intestinal microbiota mediated metabolism of voglibose in vitro

For time and concentration dependent metabolism of voglibose, 10 µl of either 0.25, 0.5 or 1 mg/ml voglibose was incubated with 490 µl of intestinal contents to prepare the final concentrations of 5, 10 and 20 µg/ml of voglibose. The mixtures were incubated under an

anaerobe gas generating system at 37°C for 3, 6, 12 and 24 hr. Following incubation, 10 µl of each incubation mixture was transferred to another tube containing 490 µl of methanol containing 10 ng/ml of telmisartan (IS), so that the final concentration for LC-MS/MS analysis could be 100, 200 and 400 ng/ml. The mixture was vortexed and centrifuged at 12,000 g for 10 min. Then, 200 µl of supernatant was pipetted into vials and analyzed by LC-MS/MS. Calibration curves were prepared accordingly and concentrations of voglibose in incubated samples from antibiotics- and vehicle-treated mice were determined.

2.9. Pharmacodynamics of voglibose in non-diabetic mice

To investigate the role of intestinal microbiota on pharmacodynamics of voglibose in non-diabetic conditions, mice were divided into antibiotics-treated and vehicle-treated groups. Antibiotics-treated animals were administered orally with the mixture of 3 antibiotics as mentioned above for three successive days. Saline was administered in vehicle-treated animals for three days. After 24-hr of last treatment with antibiotics, both antibiotics- and vehicle-treated mice were sub-divided into two subgroups as starch+voglibose and starch only group, followed by fasting period of 6-hr. For the main experiment, voglibose at 10 mg/kg was administered orally to starch+voglibose group and saline to starch only group. Thirty min later, 50 mg/kg soluble starch was orally administered to all animals and the blood glucose level was determined thereafter at 0, 30, 60, 90, 120, 180 and 240 min after starch administration.

2.10. Pharmacodynamics of voglibose in diabetic mice

To prepare diabetic model, 6-hr fasted mice were intraperitoneally injected with 40 mg/kg streptozotocin for five successive days. Body weight and blood glucose level of individual animals were measured daily before each streptozotocin injection. Following blood glucose level reached to > 250 mg/dL, mice were considered as diabetic and divided into two groups

for antibiotics treatment. Mice in antibiotics-treated group were administered orally with the mixture of 3 antibiotics as mentioned above for three consecutive days. In vehicle-treated group, mice were administered with saline for three days. After 24-hr of last dose of antibiotics, mice were further divided into two groups as starch+voglibose group and starch only group. For the main experiment, voglibose at 10 mg/kg was administered to starch+voglibose group and saline was administered to starch only group to 6-hr fasted animals. Thirty min later, 50 mg/kg soluble starch was orally administered to all mice in both groups for the elevation of blood glucose, which was measured at 0, 30, 60, 90, 120, 180 and 240 min after starch administration.

2.11. Statistics

All data were presented as mean ± standard deviation (SD). Student's t-test was used to determine the statistical significance of data. The results of $p < 0.05$ were represented as an asterisk.

3.0 Results

3.1. Development and optimization of analytical method

For the accurate quantification of voglibose, an LC-MS/MS method was developed. The separation of voglibose was better with C_8 column than with C_{18} or C_{16} columns (data not shown). In addition, for better resolution, a gradient eluting system was established. As depicted in Fig. 1A, voglibose was ionized at positive ion mode $[M+H]^+$ to form a protonated ion $[M+H]^+$ at *m/z* 268.0 with a daughter ion at *m/z* 92.0. Telmisartan, an IS, was detected at

m/z 515.3 and further fragmented to the ion of *m/z* 276.2 (Fig. 1B). There was no interfering peak at the retention time of voglibose at 1.4 min and telmisartan at 7.93 min (Fig. 2). The inter- and intra-day accuracy and precision of voglibose are summarized in Table 1. A good linearity was observed with the calibration curves constructed with the ratio of areas of analyte to IS peaks. Acceptable linearity was observed between the constructed calibration standards with 4 ng/ml - 400 ng/mL voglibose. The accuracy (%) and CV (%) of inter-day and intra-day were within 15% for the lowest concentration and 10% for other concentrations tested.

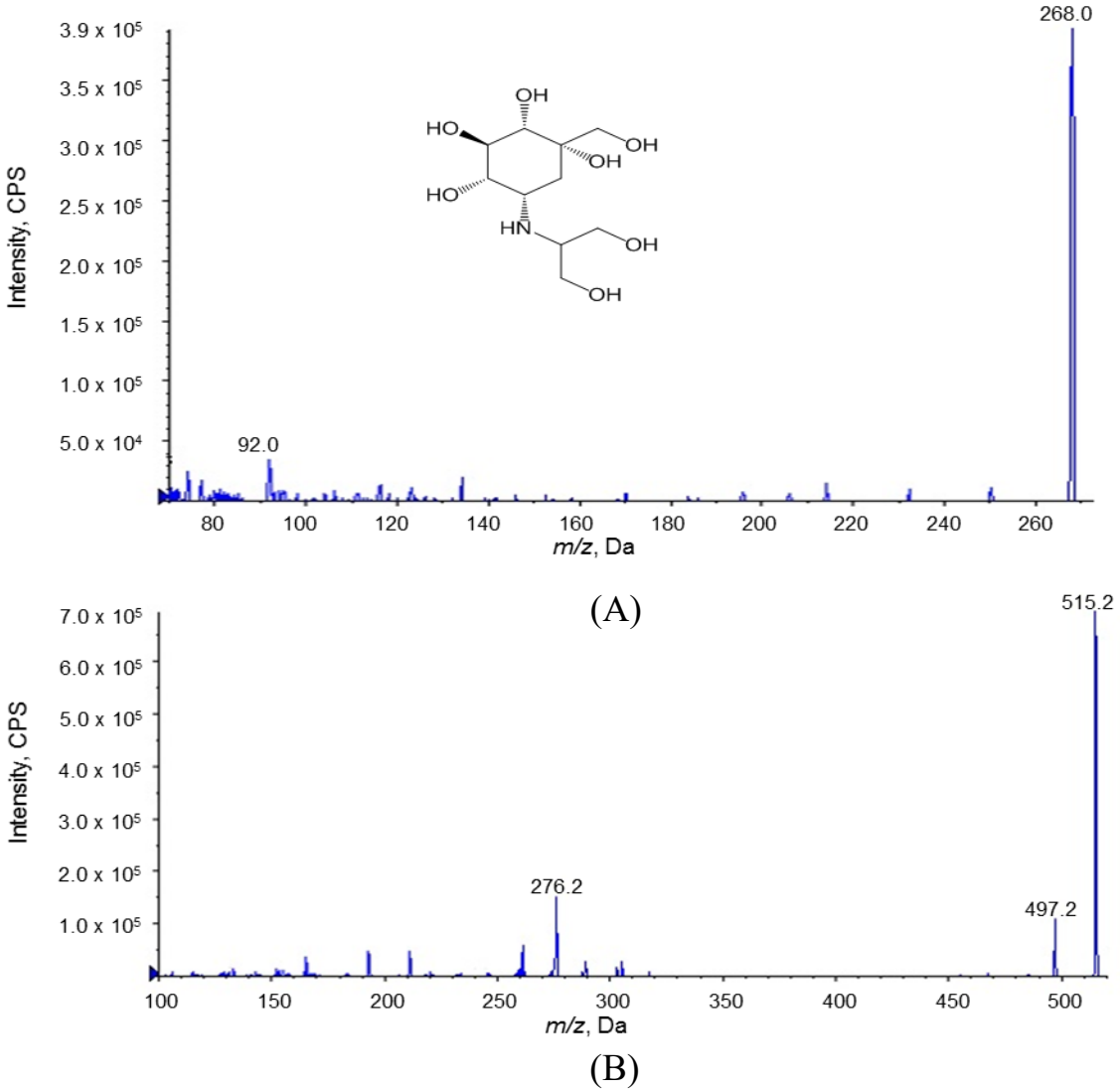


Figure 1. Mass spectra of (A) voglibose with its structure and (B) telmisartan, an internal standard.

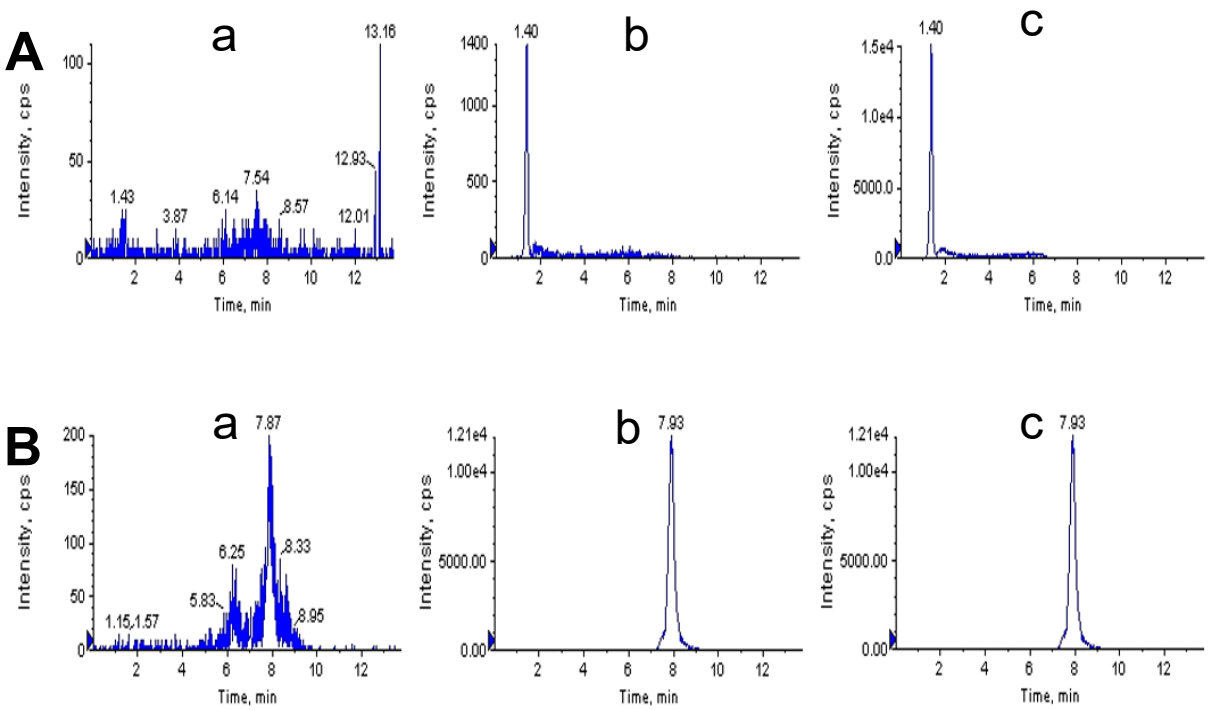


Figure 2. Representative LC-MS/MS chromatograms of (A) voglibose and (B) telmisartan. (a) blank samples; (b) blank samples spiked with analyte at LLOQ; and (c) real samples from *in vitro* incubation with intestinal contents for 3 hr.

Table 1. Method validation

Voglibose (ng/ml)	Inter-day (n=5)		Intra-day (n=5)	
	Accuracy (%)	CV (%)	Accuracy (%)	CV (%)
4	108.0 ± 9.3	8.6	85.7 ± 5.9	6.9
100	104.1 ± 6.7	6.5	99.0 ± 6.8	6.9
400	107.8 ± 9.5	8.8	99.4 ± 8.1	8.2

In addition, stability test was conducted for three different concentrations of voglibose. Following exposure of voglibose to various ambient conditions, LC-MS/MS analysis was conducted to measure the remained voglibose from the initial level. As shown in Table 2, voglibose was stable for 24 hr at both room temperature and 37°C. Similarly, voglibose was stable under freezing and refrigerating conditions. However, voglibose was lesser stable in

samples with freeze-thaw cycle for at least 3 times. Nevertheless, it was within the limit of 15%. Taken together, the current analytical method would be appropriate to study *in vitro* metabolism of voglibose by intestinal microbiota.

Table 2. Stability of voglibose under ambient conditions.

Ambient conditions	Voglibose remained (%)		
	0.1 μg/ml	0.2 μg/ml	0.4 μg/ml
Short-term at 25°C	96.3 ± 11	97.5 ± 5.7	94.4 ± 4.6
Short-term at 37°C	87.8 ± 4.0	95.9 ± 1.2	99.2 ± 3.8
Long-term at +4°C	95.1 ± 8.8	96.6 ± 0.8	96.6 ± 2.4
Long-term at -20°C	99.0 ± 8.8	98.2 ± 5.6	96.9 ± 7.0
Freeze-thaw cycle (-20°C to 25°C)	89.5 ± 2.1	91.4 ± 11.1	94.6 ± 1.9

Short-term stability tests were conducted at 25°C and 37°C for 24 hr. Long-term stability tests were conducted after storing voglibose in freezer and refrigerator (+ 4°C) for 30 and 5 days, respectively. Three freeze-thaw cycles were tested for freeze-thaw stability tests from −20°C to room temperature. Each value represents the mean percent of voglibose remained ± S.D. of triplicate samples.

3.2. In vitro metabolism of voglibose

Prior to the characterization of voglibose metabolism by intestinal microbiota, three enzyme activities, i.e., β-D-glucosidase, β-D-glucuronidase and sulfatase, of intestinal contents isolated from both vehicle- and antibiotics-treated mice were determined. As shown in Fig. 3, the enzyme activities in antibiotics-treated mice was significantly lower than in vehicle-treated mice, indicating that the present antibiotics pre-treatment model would be effective to study the role of intestinal microbiota in voglibose metabolism and their effects on pharmacodynamics of voglibose.

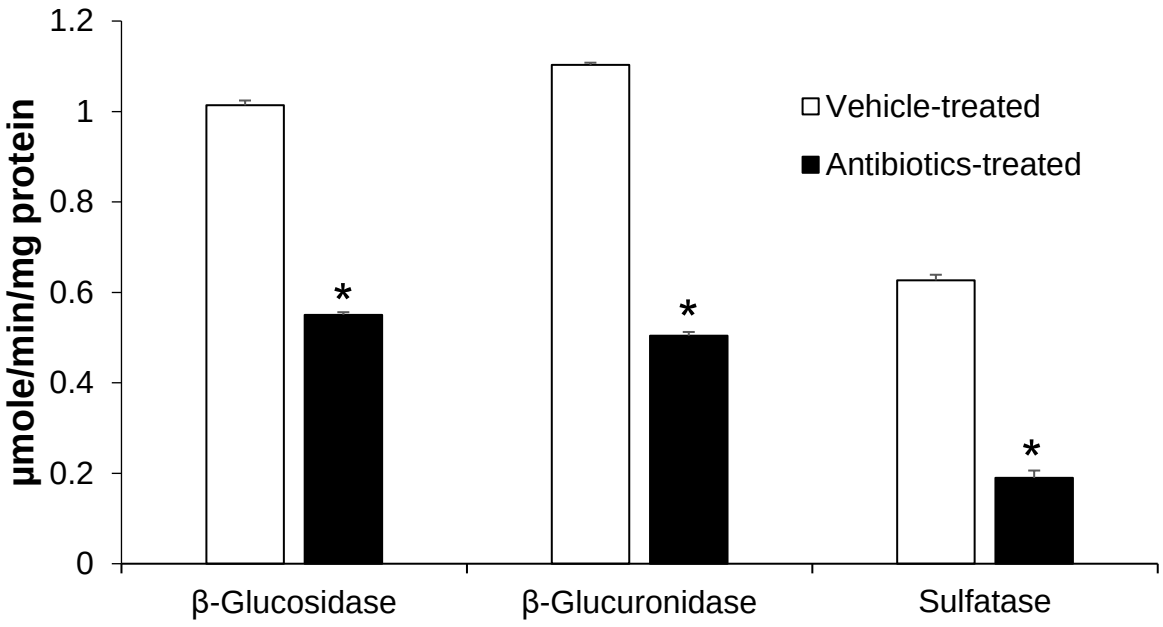


Figure 3. Enzyme activities of intestinal contents. 0.4 ml of either 2.5 mM 4-nitrophenyl β-D-glucopyranoside, 4-nitrophenyl β-D-glucuronide, or 4-nitrophenyl-sulfate in potassium phosphate buffer, pH 7.4, was mixed with 0.4 ml of potassium phosphate buffer, and 0.2 ml of intestinal contents prepared from both antibiotics- and vehicle-treated mice and incubated at 37°C for 30 min. The enzyme activities were calculated following measuring the sample's absorbance at 405 nm. Each bar represents the mean activity of respective enzymes + S.D. of triplicate determinations. An asterisk indicates the value significantly different from corresponding vehicle-treated controls at $p < 0.05$.

Thereafter, several concentrations of voglibose were incubated with intestinal contents from vehicle- and antibiotics-treated mice for several time points. And then, the remained voglibose was measured. Following incubation, almost 40% of the voglibose in control was disappeared within 3 hr of incubation when compared to the level of voglibose in antibiotics-treated group. Moreover, the level of voglibose in antibiotics-treated group was always higher than the level of voglibose in control up to the period of sampling tested (Fig. 4). The results indicated that the intestinal microbiota might play a critical role in metabolism of voglibose. In addition, a small but gradual increase in metabolism of voglibose over time in antibiotics-treated group indicated the reduced number of bacteria in intestinal contents

following antibiotics treatment to mice. The results indicated that the pharmacodynamic action of voglibose would be significantly modulated by the condition of intestinal microbiota. To support this hypothesis, *in vivo* pharmacodynamic action of voglibose was investigated in a conditionally modulated intestinal microbiota by antibiotics treatment to mice.

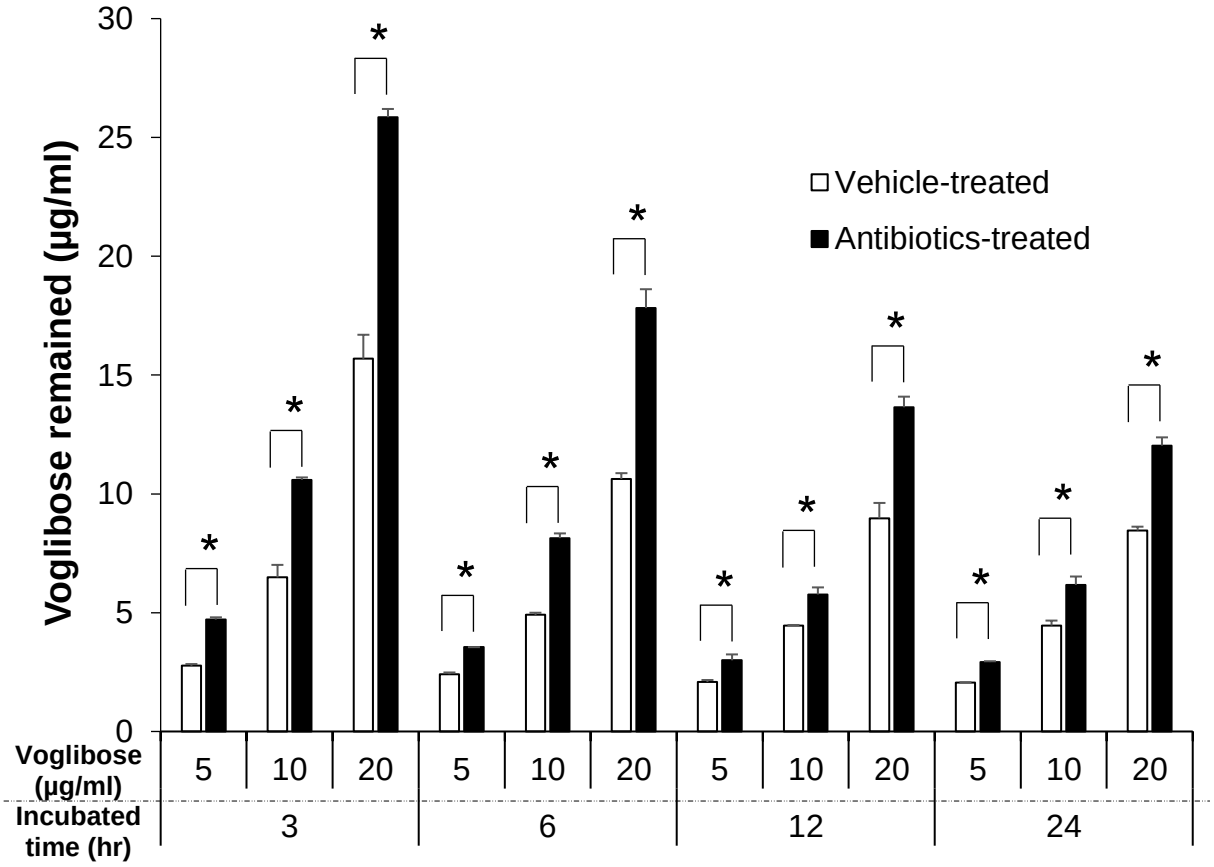


Figure 4. Time- and concentration-dependent metabolism of voglibose by intestinal microbiota *in vitro*. Various concentrations of voglibose was incubated with 0.5 g/ml intestinal contents prepared from both antibiotics- and vehicle-treated mice and incubated at 37°C for various time points. The remained voglibose over time was measured by LC-MS/MS. Each bar represents the mean concentration of voglibose in the incubated sample + S.D. of triplicate determinations. An asterisk indicates the value significantly different from corresponding vehicle-treated controls at $p < 0.05$.

3.3. Pharmacodynamics of voglibose in normal and diabetic mice

Because antibiotic pretreatment to mice would cause the reduction of intestinal microbial population, the enzymes available for voglibose metabolism would be reduced and, thereby, the metabolism of voglibose by intestinal microbiota would be altered. Due to the low intestinal absorption of voglibose, pharmacodynamic effects of voglibose in starch-supplemented animals were investigated in the present study. To illustrate the effect of intestinal microbiota in the metabolism of voglibose *in vivo,* the effect of voglibose for its proficiency to lower blood glucose level was measured in vehicle- and antibiotics-treated non-diabetic and diabetic mice models (Fig. 5A and 5B). The blood glucose level was rapidly increased following starch administration to 6-hr fasted animals. In addition, the baseline level of blood glucose was much higher in diabetic mice than that of non-diabetic normal mice. Meanwhile, following voglibose administration in both antibiotics- and vehicle-treated mice of both non-diabetic and diabetic mice models, the blood glucose level even after starch administration was precisely controlled and was similar to the level at 0-hr time point from 2 hr time point. Particularly, a significant difference in blood glucose level was achieved between voglibose-administered antibiotics- and voglibose administered vehicle-treated groups of non-diabetic model (Fig. 5A). The levels of blood glucose in antibiotics-treated group were also well attenuated by voglibose in diabetic mice, although the results were not statistically significant (Fig. 5B). The results suggested that voglibose could completely control the blood glucose level by inhibiting α-glucosidase enzymes present in the intestine, particularly in antibiotic-treated animals. The results might be due to the limited availability of microbial enzymes to metabolize voglibose by antibiotics administration, by which more voglibose could show its effect as a blood glucose-controlling agent via the inhibition of α-glucosidase enzyme.

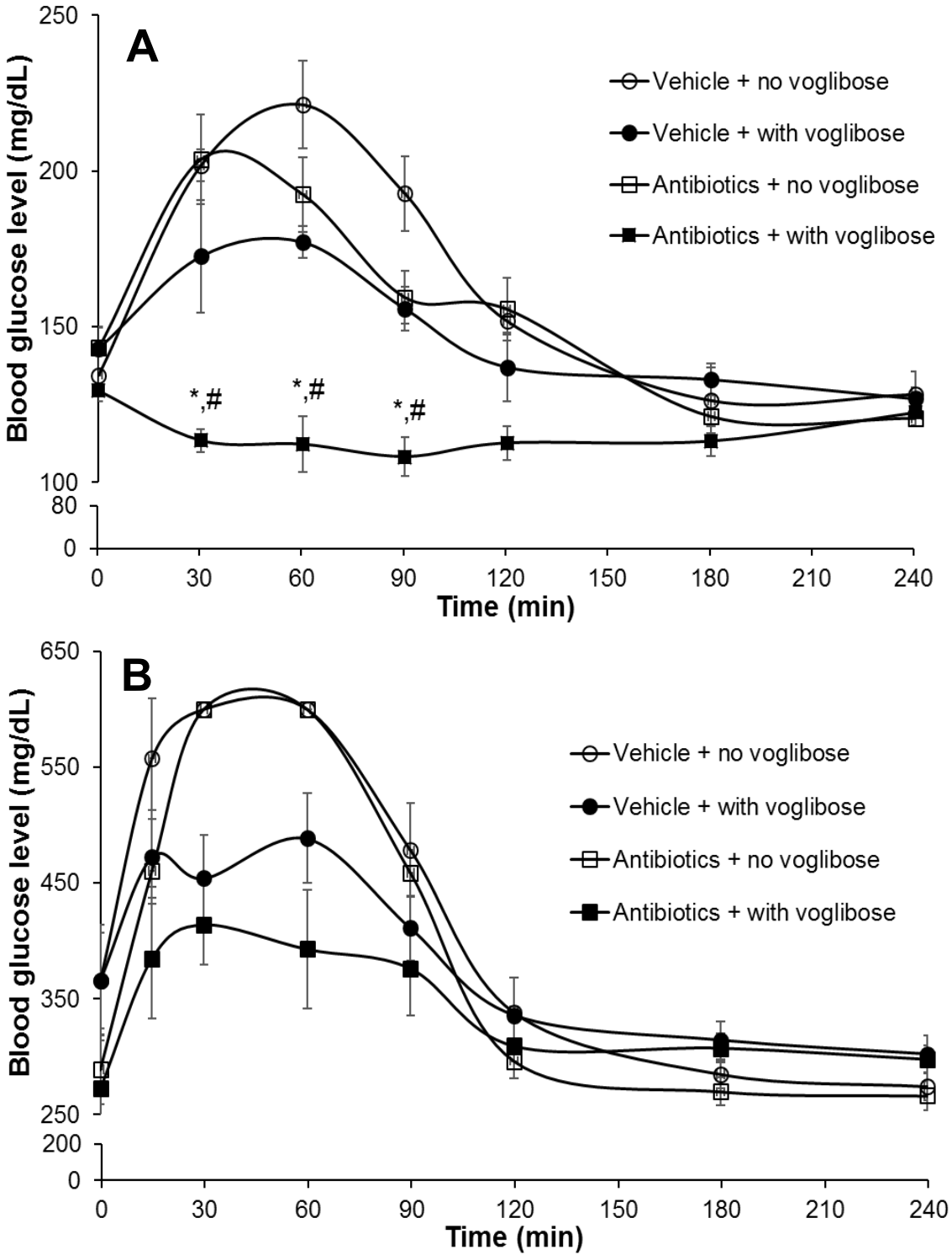


Figure 5. Blood glucose level in vehicle-treated and antibiotics-treated non-diabetic normal mice (A) and diabetic mice (B). Six-hr fasted vehicle- and antibiotics-treated non-diabetic and diabetic mice were administered with voglibose followed by an oral administration with starch. And then, the change in the blood glucose level was measured at various time points. Each point represents mean blood glucose level ± S.D. of five animals. An asterisk (*) indicates

significant difference between antibiotics-treated starch only and antibiotics-treated starch+voglibose groups. A hash (#) indicates significant difference between vehicle-treated starch+voglibose and antibiotics-treated starch+voglibose groups.

4.0 Discussion

Drug metabolism study is a broad subject that covers not only liver enzymes-mediated metabolism, but also gut microbiota-mediated metabolism [12]. Since few decades, studies have extensively been carried out to determine the role of intestinal microbiota in drug metabolism. From the studies, now it is generally accepted that metabolism by intestinal microbiota would be important not only for the formation of new metabolites that cannot be produced in livers but also alteration of pharmacokinetics and pharmacodynamics of many drugs, including amiodarone, amlodipine, aspirin, and lovastatin [8,10,19].

Voglibose has a negligible intestinal absorption property and no liver metabolites were identified yet [15]. In the intestinal lumen, by inhibiting α-glucosidase, complex carbohydrates could not be broken down effectively, by which glucose absorption would be delayed. In our preliminary study, it was noticed that voglibose could be metabolized by intestinal microbiota, although the exact metabolites were not identified (Fig. 4). The level of voglibose was dramatically reduced by the incubation with intestinal contents prepared from control mice that contain normal microflora. Therefore, to further explore the action of intestinal microbiota on voglibose metabolism *in vivo*, pseudo-germ free animals were prepared by antibiotic pre-treatment.

For the accurate preparation of pseudo-germ free animals, several *in vivo* antibiotics-treated models have been employed [13,20,21]. Because gut microflora consisted of both

Gram-positive and Gram-negative bacteria, researchers to prepare pseudo-germ free mice have used mixtures of broad-spectrum antibiotics. In our previous study, an antibiotic treatment model using the mixture of oxytetracycline, erythromycin and cefadroxil was employed and tested the ability of the mixture to reduce the number of bacteria in separated intestinal contents from small intestine, cecum, and large intestine [21]. The results showed that numbers of bacteria in intestinal contents collected from all three parts were significantly lowered by antibiotic treatment. Therefore, the same schedule for antibiotic treatment was employed in the current study. Following antibiotic administration, at first, the efficacy of the antibiotic treatment was tested by determining various enzyme activities produced by intestinal microbiota (Fig. 3). As results, significant decreases in the enzyme activities of intestinal contents were achieved by antibiotic treatment. These results indicated that the present antibiotics treatment model would be effective for studying the possible role of intestinal microbiota in voglibose metabolism.

Thereafter, *in vitro* metabolism of voglibose was characterized by incubating various concentrations of voglibose for various time points with the intestinal contents prepared from antibiotics- and vehicle-treated mice. As shown in Fig. 4, the amount of voglibose was significantly reduced within 3 hr of incubation in control, when compared with the results from antibiotic-treated group, up to 24 hr. To confirm, whether the decrease in the level of voglibose in 24-hr incubated samples was due to the enzymatic action of intestinal microbiota or because of the unstable condition of voglibose at 37°C, the stability test for voglibose was also conducted. The stability results in Table 2 demonstrated that the disappearance of voglibose was due to the action by intestinal microbiota, but not by the stability related issues of voglibose at 37°C for 24 hr. A gradual disappearance of voglibose from intestinal contents prepared from

antibiotic-treated mice also indicated that the pre-treatment mice with oral antibiotics could not absolutely suppress the enzymes from intestinal microbiota [21].

From previous study, it has been clear that voglibose would be neither adequately absorbed nor metabolized by liver enzymes [15]. Therefore, it was impractical to perform the pharmacokinetic study of voglibose in animals. Because voglibose acts as a blood glucose-lowering agent by inhibiting the breakdown of complex carbohydrates in the intestinal lumen, it would be reasonable to observe the pharmacological effects of voglibose following antibiotic pretreatment to mice in the starch-supplemented model. Therefore, the alteration of blood glucose level by voglibose was studied in antibiotic-pretreated non-diabetic and diabetic mice with their respective control groups.

Because voglibose is originally developed as a blood sugar lowering agent, it would normally be prescribed to the diabetic patients rather than normal patients. Therefore, it would be more substantial to perform the voglibose pharmacodynamic study in diabetic mice model. Therefore, the role of intestinal microbiota in metabolism of voglibose was also tested in diabetic mice in the present study. To prepare the diabetic mice model, several methods are available elsewhere [22-25]. Preparation of type I diabetic mice using streptozotocin is one of the simplest and widely accepted methods [23]. From a preliminary study, we found that single streptozotocin dose higher than 200 mg/kg could destroy pancreatic β cells rapidly within 1 or 2 days, but we found that it was very difficult to control the blood glucose level and mice died within 10 days of streptozotocin injection (data not shown). Although the result was consistent with a report, where the administration of animals with high dose of streptozotocin yielded complete death of β cells and a state of complete absence of insulin was achieved [26], this was not appropriate for our study purposes. Meanwhile, literatures indicated that multiple low doses

of streptozotocin caused delayed onset of hyperglycemia because of the partial damages to the pancreatic islets cells and, thereby, the blood glucose level in mice could be adequately controlled [27,28]. Therefore, we employed multiple dose of streptozotocin at lower level and observed that, with the intraperitoneal injection of mice with 40 mg/kg streptozotocin for consecutive 5 days, blood glucose level was elevated to > 250 mg/dL, which was considered as the diabetic condition in the present study [27].

Subsequently, *in vivo* pharmacodynamic study of voglibose was performed in antibiotics- and vehicle-treated non-diabetic and diabetic mice. It was hypothesized that, due to the diminished metabolism of voglibose by antibiotic treatment, the blood glucose-controlling activity of voglibose would be significantly elevated and would be more pronounced in antibiotic-treated animals. As expected, following voglibose and starch administration to mice of non-diabetic model, the elevation in blood glucose level was precisely controlled and was similar to the 0-min values (Fig. 5A). Conversely, the blood glucose level was highly elevated in starch-supplemented groups in absence of voglibose. A significant difference was achieved between the blood glucose levels of voglibose administered antibiotics- and vehicle-treated non-diabetic mice. These results indicated that the metabolism of voglibose was dependent on intestinal microbiota, which was largely altered following antibiotic treatment. The same study was also conducted in diabetic mice and the results were similar. Although the patterns of blood glucose level in mice were similar in both non-diabetic and diabetic mice model, no statistically differences were achieved between the groups, probably because of the large blood-glucose level variation in diabetic model. The difference in blood glucose level between antibiotics-treated and -untreated groups proved that the antibiotics pretreatment to mice decreased in the intestinal microbial population, and thereby, resulted in the decreased microbial enzyme levels to reduce the metabolism of voglibose. Lesser metabolism of voglibose refers to the availability

of more voglibose to show its effect as a blood glucose-controlling agent. Hence, the lowest blood glucose level was observed in antibiotics-pretreated mice administered with voglibose in both diabetic and non-diabetic mice models. Following antibiotic treatment to mice, blood glucose level in the basal level was observed to be slightly lesser than in vehicle-treated group, which was also observed even after starch administration in antibiotic-treated group at each time point. Similar results were observed, when antibiotic-induced microbiome depletion caused disturbance in gut homeostasis, luminal signaling, and metabolism, by which the baseline blood glucose level was reduced [29].

Taken together, it was concluded that the metabolism of voglibose would be significantly mediated by intestinal microbiota, by which the pharmacodynamic effects of voglibose could be modulated when antibiotics were pretreated to mice. To our knowledge, the role of gut microbiota in voglibose metabolism and the subsequent changes in pharmacodynamic effects was demonstrated for the first time. Because the results referred to the pharmacodynamic interactions of voglibose and antibiotics, a clinical study to determine whether the concurrent administration of voglibose and antibiotics to patients should be investigated to demonstrate whether the disturbance in the metabolism of voglibose can produce the modulation of blood glucose level as in the animal models. In addition, further studies are needed to identify the possible metabolites produced by intestinal microbiota in the near future.

Author Contributions

Conceptualization, M.R.N. and T.C.J.; Data curation, M.R.N.; Formal Analysis, M.J.K., G.H.K., D.H.C., and J.S.K.; Investigation, M.R.N., M.J.K., D.H.C., and J.S.K.; Validation, J.H.K.; Funding Acquisition, T.C.J.; Supervision, T.C.J.; Writing-Original Draft Preparation, M.R.N.; Writing-Review & Editing, J.H.K. and T.C.J.

Conflicts of interest

The authors declare no conflict of interest.

Funding

This research was supported by the grant from National Research Foundation, Korea (NRF-2017RIDIA3B04033313).

References

1. O'Hara, A.M.; Shanahan, F. The gut flora as a forgotten organ. *EMBO reports* **2006**, *7*, 688-693.
2. Scarpellini, E.; Ianiro, G.; Attili, F.; Bassanelli, C.; De Santis, A.; Gasbarrini, A. The human gut microbiota and virome: Potential therapeutic implications. *Dig. Liver Dis.* **2015**, *47*, 1007-1012.
3. Hur, K.Y.; Lee, M.S. Gut microbiota and metabolic disorders. *Diabetes Metab. J.* **2015**, *39*, 198-203.
4. Thursby, E.; Juge, N. Introduction to the human gut microbiota. *Biochem. J.* **2017**, *474*, 1823-1836.
5. Liang, D.; Leung, R.K.; Guan, W.; Au, W.W. Involvement of gut microbiome in human health and disease: brief overview, knowledge gaps and research opportunities. *Gut pathog.* **2018**, *10*, 3.
6. Doré, J.; Simrén, M.; Buttle, L.; Guarner, F. Hot topics in gut microbiota. *United European Gastroenterol. J* **2013**, *1*, 311-318.
7. Gavhane, Y.N.; Yadav, A.V. Loss of orally administered drugs in GI tract. *Saudi Pharm. J.* **2012**, *20*, 331-344.
8. Kang, M.J.; Kim, H.G.; Kim, J.S.; Oh, D.G.; Um, Y.J.; Seo, C.S.; Han, J.W.; Cho, H.J.; Kim, G.H.; Jeong, T.C., Jeong, H.G. The effect of gut microbiota on drug metabolism. *Expert Opin. Drug Metab. Toxicol.* **2013**, *9*, 1295-1308.
9. Pellock, S.J.; Redinbo, M.R. Glucuronides in the gut: Sugar-driven symbioses between microbe and host. *J. Biol. Chem.* **2017**, *292*, 8569-8576.

10. Noh, K.; Kang, Y.R.; Nepal, M.R.; Shakya, R.; Kang, M.J.; Kang, W.; Lee, S.; Jeong, H.G.; Jeong, T.C. Impact of gut microbiota on drug metabolism: an update for safe and effective use of drugs. *Arch. Pharm. Res.* **2017**, *40*, 1345-1355.

11. Jeong, H.G.; Kang, M.J.; Kim, H.G.; Oh, D.G.; Kim, J.S.; Lee, S.K.; Jeong, T.C. Role of intestinal microflora in xenobiotic-induced toxicity. *Mol. Nutr. Food Res.* **2013**, *57*, 84-99.

12. Wilson, I.D.; Nicholson, J.K. Gut microbiome interactions with drug metabolism, efficacy, and toxicity. *Transl. Res.* **2017**, *179*, 204-222.

13. Yoo, D.H.; Kim, I.S.; Van Le, T.K.; Jung, I.H.; Yoo, H.H.; Kim, D.H. Gut microbiota-mediated drug interactions between lovastatin and antibiotics. *Drug Metab. Dispos.* **2014**, *42*, 1508-1513.

14. Jiao, X.; Wang, Y.; Lin, Y.; Lang, Y.; Li, E.; Zhang, X.; Zhang, Q.; Feng, Y.; Meng, X.; Li, B. Blueberry polyphenols extract as a potential prebiotic with anti-obesity effects on C57BL/6 J mice by modulating the gut microbiota. *J. Nutr. Biochem.* **2019**, *64*, 88-100.

15. Dabhi, A.S.; Bhatt, N.R.; Shah, M.J. Voglibose: an alpha glucosidase inhibitor. *J. Clin. Diagn. Res.* **2013**, *7*, 3023-3027.

16. Oh, T.J.; Yu, J.M.; Min, K.W.; Son, H.S.; Lee, M.K.; Yoon, K.H.; Song, Y.D.; Park, J.Y.; Jeong, I.K.; Cha, B.S., Kim, Y.S., Baik, S.H., Kim, I.J., Kim, D.M., Kim, S.R., Lee, K.W., Park, J.H., Lee, I.K., Park, T.S., Choi, S.H., Park. S.W. Efficacy and safety of voglibose plus metformin in patients with type 2 diabetes mellitus: a randomized controlled trial. *Diabetes Metab. J.* **2018**, *42*, 1-11.

17. Mozes, S.; Sefcikova, Z.; Bujnakova, D.; Racek, L. Effect of antibiotic treatment on intestinal microbial and enzymatic development in postnatally overfed obese rats. *Obesity* **2013**, *21*, 1635-1642.

18. USFDA. Guidance for industry on bioanalytical method validation. 2001. *URL: http://www. fda. gov/downloads/Drugs/Guidances/ucm070107.pdf (accessed 10.05. 2019)*.

19. Kim, D.H. Gut microbiota-mediated drug-antibiotic interactions. *Drug Metab. Dispos.* **2015**, *43*, 1581-1589.

20. Hertz, F.B.; Løbner-Olesen, A.; Frimodt-Møller, N. Antibiotic selection of *Escherichia coli* sequence type 131 in a mouse intestinal colonization model. *Antimicrob. Agents Chemother.* **2014**, *58*, 6139-6144.

21. Kang, M.J.; Ko, G.S.; Oh, D.G.; Kim, J.S.; Noh, K.; Kang, W.; Yoon, W.K.; Kim, H.C.; Jeong, H.G.; Jeong, T.C. Role of metabolism by intestinal microbiota in pharmacokinetics of oral baicalin. *Arch. Pharm. Res.* **2014**, *37*, 371-378.

22. Graham, M.L.; Janecek, J.L.; Kittredge, J.A.; Hering, B.J.; Schuurman, H.J. The streptozotocin-induced diabetic nude mouse model: differences between animals from different sources. *Comp. Med.* **2011**, *61*, 356-360.

23. Wu, J.; Yan, L.J. Streptozotocin-induced type 1 diabetes in rodents as a model for studying mitochondrial mechanisms of diabetic β cell glucotoxicity. *Diabetes Metab. Syndr. Obes.* **2015**, *8*, 181-188.

24. King, A.J. The use of animal models in diabetes research. *Br. J. Pharmacol.* **2012**, *166*, 877-894.

25. Van Belle, T.L.; Taylor, P.; von Herrath, M.G. Mouse Models for Type 1 Diabetes. *Drug Discov. Today Dis. Models* **2009**, *6*, 41-45.

26. Furman, B.L. Streptozotocin-induced diabetic models in mice and rats. *Curr. Protoc. Pharmacol.* **2015**, *70*, 5.47, 1-20.

27. Deeds, M.C.; Anderson, J.M.; Armstrong, A.S.; Gastineau, D.A.; Hiddinga, H.J.; Jahangir, A.; Eberhardt, N.L.; Kudva, Y.C. Single dose streptozotocin-induced

diabetes: considerations for study design in islet transplantation models. *Lab. Anim.* **2011**, *45*, 131-140.

28. Like, A.A.; Rossini, A.A. Streptozotocin-induced pancreatic insulitis: new model of diabetes mellitus. *Science* **1976**, *193*, 415-417.

29. Zarrinpar, A.; Chaix, A. Antibiotic-induced microbiome depletion alters metabolic homeostasis by affecting gut signaling and colonic metabolism. *Nature Commun.* **2018**, *9*, 2872.