Original Article

Construction of a simple evaluation system for the intestinal absorption of an orally administered medicine using *Bombyx mori* larvae

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Summary

Human intestinal absorption is estimated using a human colon carcinoma cell line (Caco-2) cells from human colorectal adenocarcinoma, intestinal perfusion, or a mammalian model. These current evaluation systems are limited in their ability to estimate human intestinal absorption. In addition, *in vivo* evaluation systems using laboratory animals such as mice and rats entail animal ethics problems, and it is difficult to screen compounds on a large scale at the drug discovery stage. Thus, we propose the use of *Bombyx mori* larvae for evaluation of intestinal absorption of compounds as an alternative system in this study. First, to compare the characteristics among Caco-2 cells, human intestine, and *B. mori* larval midgut, we analyzed their RNA-seq data, and we found 26 drug transporters common to humans and *B. mori*. Next, we quantitatively developed an oral administration technique in *B. mori* and established a method using silkworm *B. mori* larvae that can easily estimate the intestinal permeability of compounds. Consequently, we could determine the dose and technique for oral administration in *B. mori* larvae. We also developed a *B. mori* model to evaluate the intestinal permeability of orally administered. Our constructed evaluation system will be useful for evaluating intestinal permeability in medical drug development.

Keywords: Intestinal permeability, transporters, next-generation sequencing, *B. mori*

1. Introduction

Although drug discovery can be genome-based, many candidate compounds are rejected in clinical trials for pharmaceutical products. A major reason for such as rejection is poor pharmacokinetics; in particular, poor intestinal absorption of candidate compounds. As a screening method using a human colon carcinoma cell line (Caco-2) for p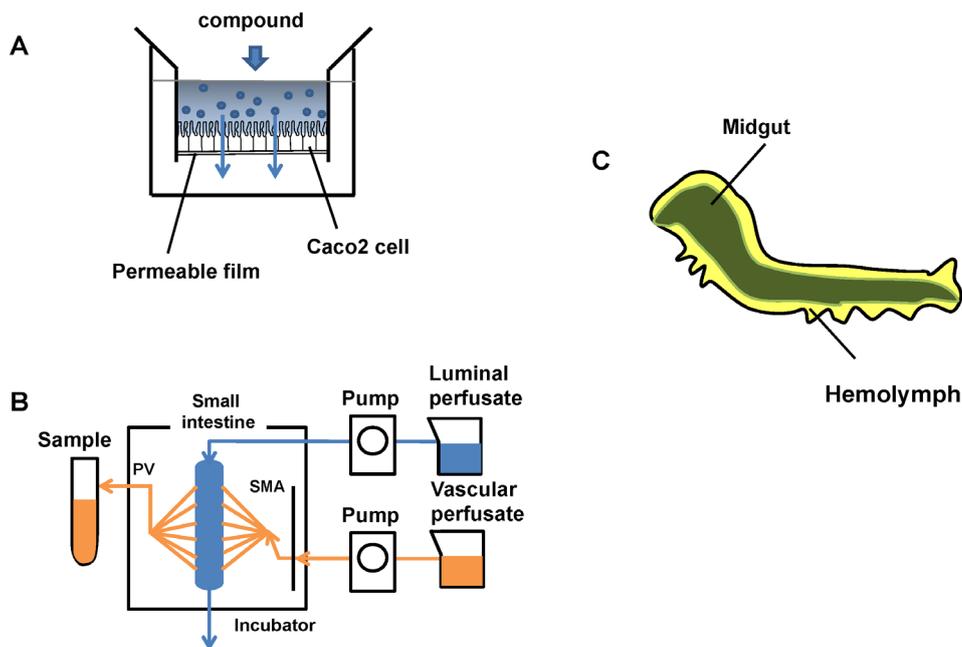redicting the intestinal absorption of a compound, the Caco-2 cell culture system is derived from high-throughput screening and *in situ* perfusion systems (Figure 1) (*1-3*). However, these current evaluation systems cannot be used to evaluate the intestinal absorption of any compound, maintaining the villus structure and function of the small intestinal epithelium (*4*). In addition, *in vivo* evaluation systems have problems related to time, expense, and the ethics of using laboratory animals, such as mice and rats. For these reasons, it is difficult to screen compounds on a large scale. Therefore, we need to construct a new evaluation method for the intestinal absorption of candidate compounds for large scale screening.

The silkworm *Bombyx mori* is a lepidopteran insect that is used as a model organism in agricultural research. Their genome sequence is almost completely

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Figure 1. Evaluation of model systems for intestinal permeability and the internal structure of *B. mori* larva. (A) Caco-2 cell system. The cells are cultured on a permeable film, and the objective compound is dissolved into the culture medium. The compound passes through the permeable film and into a lower culture dish. **(B)** Intestinal perfusion system. A modification by Ishihara *et al.* (2001) (*2*). The small intestine was dissected from mice and set into an incubator filled with buffer (37°C). A solvent containing the objective compound was perfused by a pump into the small intestine. SMA: superior mesenteric artery; PV: portal vein. **(C)** Internal structure of *B. mori* larva. Most of the body is occupied by the midgut (green), and its surroundings are filled with hemolymph (yellow).

characterized, various spontaneous genetic mutants are available, and the silkworm is amenable to transgenics, genome editing, and transcriptome analysis (*5-10*). Moreover, the larval body size is bigger than that of *Drosophila melanogaster*: their gastrointestinal tract occupies ≥ 80% of the whole larval body and is structurally similar to the human small intestine (*11*); however, *B. mori* has never been used as an evaluation model for the absorption of a compound. Hamamoto *et al.* (*12*) investigated the pharmacokinetics (absorption, distribution, metabolism, excretion) of some drugs by using silkworm larvae and showed that most of the data were consistent with those of the mouse model (*13,14*). Drugs are metabolized by P450 enzymes and are then processed in a conjugation reaction. These metabolic processes and excretion of compounds are similar to those of mammalian models (*12*); for example, the effective dose of antibiotics in the bacterial infection model using silkworm larvae was consistent with that in the mammalian model (*15*). These results indicate the possibility that silkworms can be used as a substitute for mammals.

In addition, current intestinal permeability systems cannot adequately predict the intestinal absorption of compounds at the pharmaceutical investigation stage. To evaluate pharmacokinetics without laboratory animals at the preliminary developmental stage, an evaluation system that can easily predict absorption from the small intestine of an orally administered compound is required. Such a system not only has economic advantages, such as reduced time and cost, but also improved animal ethics at the preliminary developmental stage; however, there is still no suitable screening method available. We therefore propose the use of *B. mori* larvae for the evaluation of intestinal permeability. This model can easily and conveniently predict the intestinal absorption of compounds; thus, we quantitatively developed oral administration techniques in *B. mori* and established a method using *B. mori* larvae that can rapidly measure the intestinal permeability of compounds.

2. Materials and Methods

2.1. Insects

The *B. mori* larvae used in the study were supplied by Ueda-Sha Co. Ltd. (Ueda-shi, Nagano, Japan). Silkworm larvae were reared on an artificial diet of Silkmate 2S (Nosan, Tsukuba-shi, Ibaraki, Japan). Insects were maintained at 25°C with a 12-h light/dark cycle. Day 3 fifth-instar *B. mori* larvae were used for the evaluation of intestinal absorption. The *B. mori* strain o751 (wild-type) used in the RNA-seq analysis was obtained from the Institute of Genetic Resources, Faculty of Agriculture, Kyushu University (NBRP silkworm database: *http://silkworm.nbrp.jp/index_en.html*).

2.2. *RNA-seq analysis*

Total RNA was isolated from the midgut of day 3 fifth-instar *B. mori* larvae of *B. mori* o751 wild-type by using a PureLink® RNA extraction kit (Thermo Fisher Scientific Inc., Valencia, CA, USA) according to the manufacturer's protocol. The RNA quality was assessed by using an Agilent Bio-analyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). Paired-end sequencing cDNA libraries were constructed with 4 μg of total RNA from o751 wild-type samples (*n* = 3) by using a TruSeq RNA Sample Preparation Kit Set A (Illumina Inc., San Diego, CA, USA) according to the manufacturer's protocol. RNA-seq was performed by using a HiSeq 2500 system (Illumina Inc.). The data quality of the fastq files was verified by using the FastQC tool (Babraham Bioinformatics, *http://www.bioinformatics.babraham.ac.uk/projects/fastqc/*). The around 43-M paired-end reads (2 × 150 bp) were mapped to the reference *B. mori* genome available from the Ensembl Genome database (*16*) using the Bowtie2 program version 2.3.2 with default parameters (*17*). RNA-Seq by Expectation-Maximization software version 1.3.0 was used for the calculation of expression values in transcripts per million (TPM) (*18*).

The gene expression data for Caco-2 cells and human small intestine was obtained from the Reference Expression Dataset (*19*). Data used for the comparative analysis were "Processed expression data of all samples for CAGE human PRJDB3010 (FANTOM5)" (doi: 10.6084/m9.figshare.4028613.v4) downloaded from *https://doi.org/10.6084/m9.figshare.4028613.v4.*

2.3. *Identification of B. mori human transporter homologs by systematic BLAST search*

To identify the human transporter homolog in the silkworm, genes that were homologous to human genes were identified by performing a systematic basic local alignment search tool (BLAST) search (tblastx) with a cutoff E-value of significant homology at 10^{-10} (query: human transporter cDNA sequence; database: whole silkworm cDNA sequence set from the Ensembl database).

2.4. *Determination of dose for oral administration to B. mori larvae*

To determine the dose for oral administration, we injected several amounts of water into day 3 fifth-instar *B. mori* larvae *via* their mouthparts by using a disposable syringe (Terumo, Shibuya-ku, Tokyo, Japan) with a modified TSK 30G needle (Tochigi-seiko Co. Ltd., Utsunomiya-shi, Tochigi, Japan) as an oral probe. The tip of the 30G needle was filed by using sand paper, and the edge of the needle tip was removed for oral administration. The silkworm larvae were not fed for 24 h before the administration of the compounds. The body weights of day 3 fifth-instar *B. mori* larvae were 2.88 ± 0.16 g (mean ± SD). In this experiment, we defined "success" as survival of the larvae the day following the oral administration (for 24 h), and there were no problems in the oral injection into the larva; whereas "failure" was defined as death or other problems occurring in the course of the oral injection into the larva. We statistically calculated a correlation coefficient for the relationship between dose and larval body weight by using Microsoft Excel. In this experiment, we denoted "success" as 1 and "failure" as 0.

2.5. *Determination of the LD$_{50}$ of theophylline, tetracycline hydrochloride, and chloramphenicol in day 3 fifth-instar B. mori larvae*

Theophylline, tetracycline hydrochloride, and chloramphenicol were purchased from Wako Pure Chemical Industries, Ltd. (Chuo-ku, Tokyo, Japan). To determine the 50% of lethal dose (LD$_{50}$) in day 3 fifth-instar *B. mori* larvae, we orally injected theophylline, tetracycline hydrochloride, and chloramphenicol by using the modified oral probe mentioned above. Theophylline was dissolved in 20% dimethyl sulfoxide (Wako Pure Chemical Industries, Co., Ltd.) at 0 (control), 1, 1.25, 1.50, 1.75, and 2.00 mg/g and injected at a volume of 0.18 mL/g body weight. Tetracycline hydrochloride was dissolved in distilled water (Otsuka Pharmaceutical Co., Ltd., Naruto-shi, Tokushima, Japan) at 0 (control), 1, 1.25, 1.50, 1.75, 2.00, 2.25, and 2.50 mg/g and injected at a volume of 0.18 mL/g body weight. Chloramphenicol was dissolved in 100% ethanol (Wako Pure Chemical Industries, Co., Ltd.) at 0 (control), 0.2, 0.4, 0.6, 0.7, 0.8, and 1.0 mg/g and injected at a volume of 0.18 mL/g body weight. Each of the control groups was injected with their respective undiluted solvent.

The number of dead silkworms after 24 h was counted and the mortality rate was calculated using the formula mortality rate (%) = (X/Y) × 100, where X = dead larvae in the group and Y = total larvae in the group. To calculate the LD50, larval mortality rates were analyzed by using the Probit Analysis (*20*) option of the JMP 10.0 software package (SAS Institute Japan Ltd., Minato-ku, Tokyo, Japan). After obtaining these results, we determined the LD$_{50}$ values of theophylline, tetracycline hydrochloride, and chloramphenicol for administration to *B. mori* larvae.

Theophylline, tetracycline hydrochloride, and chloramphenicol, prepared at 1.25, 1.5, and 0.6 mg/g in each solvent, respectively, were injected into 10 larvae at a volume of 0.18 mL/g body weight. After 0.5, 1, 1.5, 2, 3, 4.5, 6, and 9 h, intact hemolymph was collected from each compound-treated larva without a reducing agent and immediately frozen at −80°C until use.

2.6. Sample preparation for evaluation of intestinal permeability

To remove protein components, one volume of methanol (Wako Pure Chemical Industries, Co., Ltd.) was added to the collected hemolymph. This was mixed well by using a vortex mixer and kept on ice for 15 min and then centrifuged at 1,000g for 15 min at 4°C. The supernatant was transferred to a new tube and filtered by using a 0.45-μm filter (ADVANTECH Co., Ltd., Bunkyo-ku, Tokyo, Japan). Finally, the adjusted sample was added to seven volumes of methanol for the analysis of intestinal permeability by high-performance liquid chromatography (HPLC).

2.7. Determination of HPLC detection conditions

The following equipment was used for the detection of intestinal permeability by HPLC analysis: an intelligent pump (JascoPU.2080Plus; JASCO Co., Ltd. Hachioji-shi, Tokyo, Japan), ultraviolet detector (UV.2077Plus, Mx.2080.31, DG, 2080.53; JASCO Co., Ltd.), and an octa decyl silyl (ODS) 18 column (25 cm × 4.6 mm I.D.; GL Science, Shinjyuku-ku, Tokyo, Japan), and a step-wise gradient mode (flow rate; 0.7 mL/min). The mobile phase used the following conditions: 5% MeOH containing 0.2% acetic acid, 100% Methanol, 100:0→60:40 for 0-15 min, and 60:40→60:40 for 15-30 min. All analyses were performed at room temperature (25°C). Chromato-PRO software (Run Time Co., Ltd., Hachioji-shi, Tokyo, Japan) was used in data processing. The elution was monitored at 273 nm (theophylline) or 275 nm (tetracycline hydrochloride and chloramphenicol).

3. Results

3.1. Identification of common drug transporters in the human intestine and B. mori larval midgut

To compare the characteristics among Caco-2 cells, the human small intestine, and the *B. mori* larval midgut, we analyzed the *B. mori* larval midgut RNA-seq data to identify the homologs of the human drug transporters expressed in the small intestine. As a result, we found 26 drug transporter homologs that were common in the *B. mori* larval midgut and human intestine. In contrast, the ATP-Binding Cassette (ABC) Subfamily A Member 13 (*ABCA13*) and *ABCB4* were expressed only in the human intestine. Moreover, Solute Carrier Family (SLC) 22 Member 15 (*SLC22A15*), *ABCA5*, *ABCB1*, *ABCG2*, and Solute Carrier Organic (*SLCO*) Anion Transporter Family Member 4 A1 (*SLCO4A1*) were not expressed in the *B. mori* larval midgut. *ABCC8*, *ABCG1*, *ABCG4*, *ABCG5*, *ABCG8*, *SLCO3A1*, *SLCO5A1*, and SV2-Related Protein (*SVOP*) were not expressed in the Caco-2 cells.

Table 1. Human transporter homologs expressed in the *B. mori* larval midgut

Gene symbol	Caco-2 (CAGE value)	Human (CAGE value)	*B. mori* (TPM value)
ABCA3	2.4	2.2	81.5
ABCA5	0.8	4.2	0
ABCA13	0	0.2	0
ABCB1	2.7	5.3	0
ABCB4	0	0.8	0
ABCB5	0	0.5	13.6
ABCB6	2.5	1.8	8.8
ABCB7	3.1	3.2	1.4
ABCB8	2.6	2.8	1.7
ABCB10	2.9	3.2	2.9
ABCC1	2.4	2.2	0.1
ABCC3	3.2	4.6	0.2
ABCC4	3.7	2.5	40.3
ABCC8	0	0.8	0.4
ABCC10	2.4	2.6	0.2
ABCD3	3.6	3.6	0.5
ABCG1	0	3.7	2.3
ABCG2	2.8	5.2	0
ABCG4	0	0.2	1.2
ABCG5	0	4.5	0.8
ABCG8	0	4.7	0.1
SLC2A8	4.2	3.2	2.4
SLC15A1	2.1	5.9	0.4
SLC15A2	0.5	0.1	17.4
SLC22A1	0	0	0.2
SLC22A3	0.2	1.5	6.2
SLC22A4	0.5	2.7	9.2
SLC22A5	2.2	3.8	1.3
SLC22A6	0	0	220.8
SLC22A12	0	0	6.0
SLC22A13	0	0.1	6.8
SLC22A15	0.1	0.8	0
SLC22A16	0	0	0.2
SLCO3A1	0	1.7	0.1
SLCO4A1	1.6	2.1	0
SLCO5A1	0	0.2	0.8
SVOP	0	0.3	0.2

Additionally, *SLC22A1*, *SLC22A6*, *SLC22A12*, *SLC22A13*, and *SLC22A16* were expressed only in the *B. mori* larval midgut (Table 1). Therefore, there were few differences in the expressed genes among *B. mori*, Caco-2, and human small intestine.

The RNA-seq reads supporting the conclusions of this article are available in the Sequence Read Archive with the following accession numbers: DRR095108, DRR095109, and DRR095110.

3.2. Evaluation of the LD_{50} values of theophylline, tetracycline hydrochloride, and chloramphenicol

To determine the dose for oral administration, we injected various amounts of water into day 3 fifth-instar *B. mori* larvae by oral administration. In this experiment, we defined success as survival of the larvae surviving until the following day and failure as death or a problem occurring in the oral injection to the larvae. We then, calculated the relationship between the amount of water and larval body weight (Figure 2, Table 2), selecting 0.18
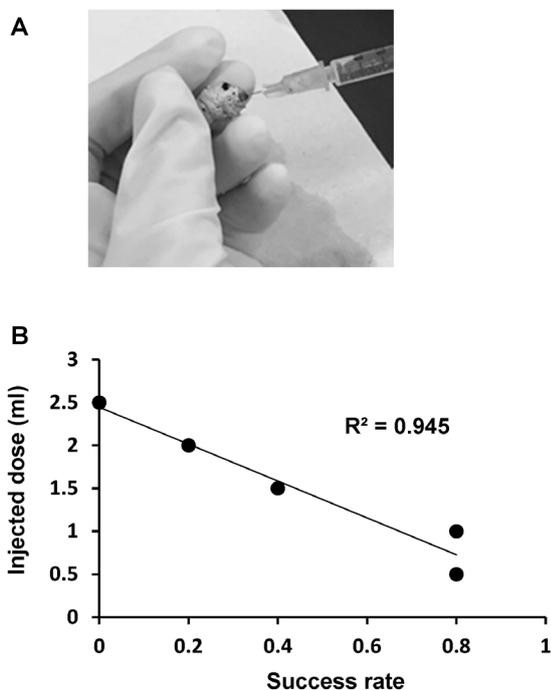
mL/g as injection dose. All oral administration doses were set at 0.18 mL/g in this study.

Next, we evaluated the LD50 of theophylline, tetracycline hydrochloride, and chloramphenicol,

A

B

Figure 2. Relationship between success rate and injection dose. (A) Oral administration to *B. mori* larvae. **(B)** Relationship between success rate and injection dose of oral administration in *B. mori* larvae. R2 indicates the correlation coefficient.

which are used as predictors of intestinal absorption in mammalian models and humans. Thus, we determined the LD50 in day 3 fifth-instar *B. mori* larvae to be 1.49 mg/g (95% confidence interval; CI, 1.37-1.60) for theophylline, 1.95 mg/g (95% CI, 1.83-2.05) for tetracycline hydrochloride, and 0.74 mg/g (95% CI, 0.69-0.80) for chloramphenicol.

3.3. *Construction of the B. mori model to evaluate the intestinal permeability of compounds by oral administration*
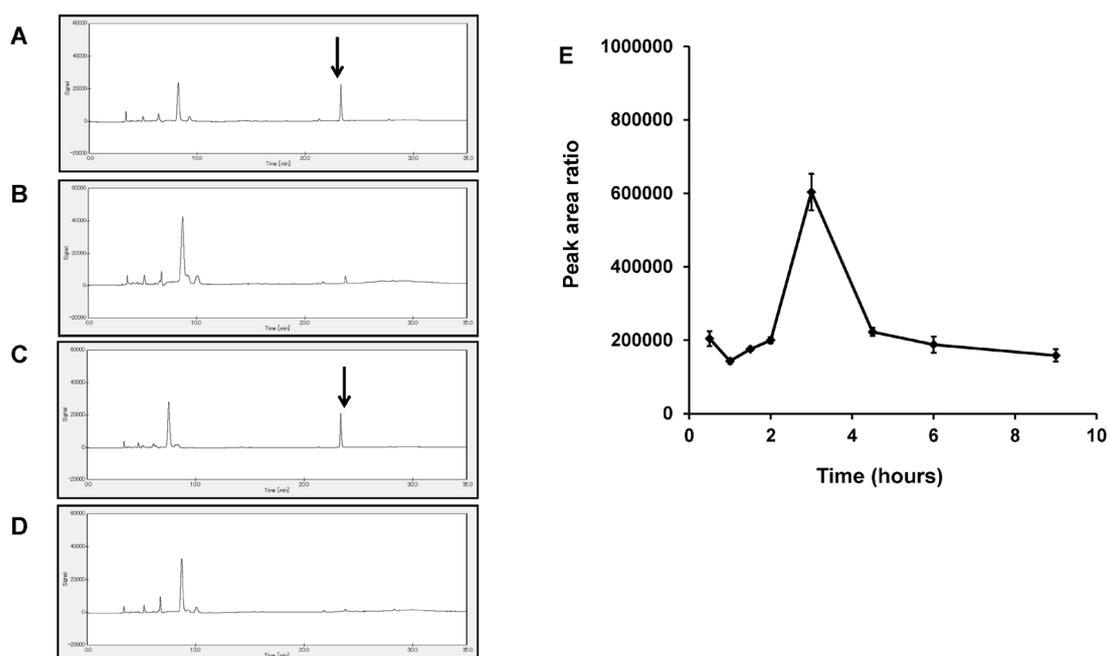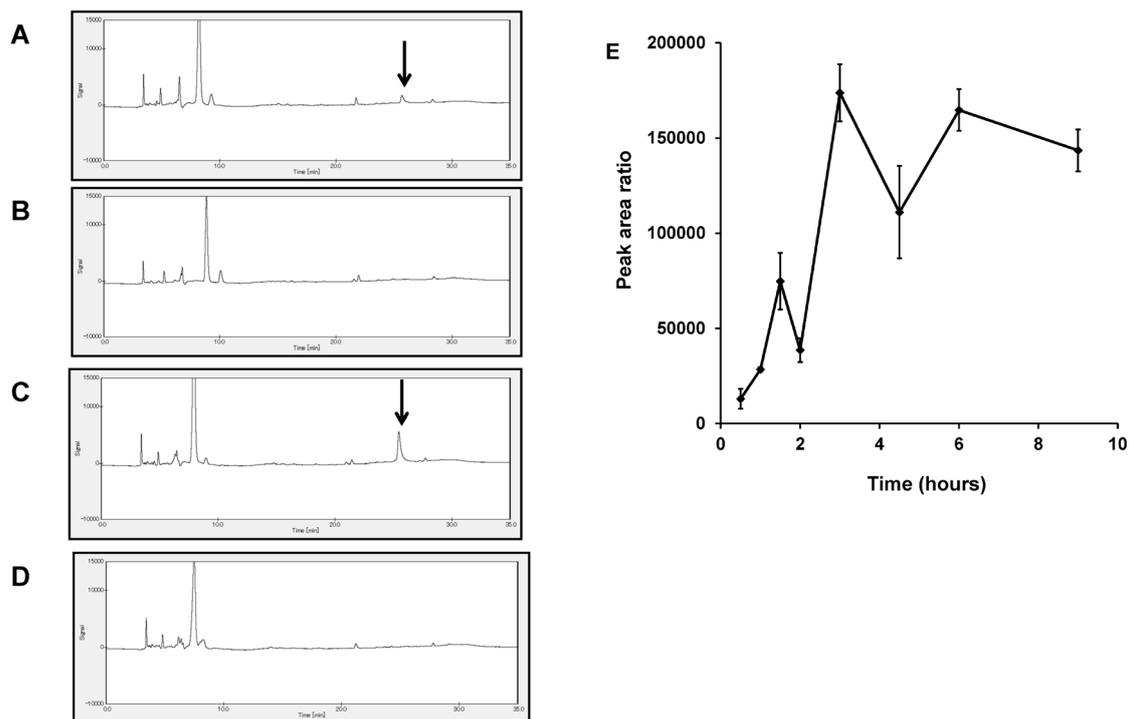
To easily evaluate the intestinal permeability of theophylline, tetracycline hydrochloride, and chloramphenicol, we assessed their intestinal permeability by using *B. mori* larvae. First, we investigated the conditions for HPLC analysis by using each compound, and the flow rate and composition of the solvent were determined. Retention times were as follows: approximately 23 min for theophylline, approximately 25 min for tetracycline hydrochloride, and 35 min for chloramphenicol. Subsequently, theophylline, tetracycline hydrochloride, and chloramphenicol were

Table 2. Relationship between success rate and injection dose

Success rate (%)	Injection dose (mL) per individual	Injection dose (mL) per body weight
80	0.73	0.25
90	0.51	0.18
95	0.40	0.14

A

B

C

D

E

Figure 3. Determination of the intestinal permeability of theophylline by HPLC analysis. (A) Oral administration of theophylline after 30 min. **(B)** Oral administration of the solvent after 30 min. **(C)** Oral administration of theophylline after 1.5 h. **(D)** Oral administration of the solvent after 1.5 h. **(E)** Time course of the detection of intestinal permeability of theophylline by HPLC analysis. The peak area ratio was calculated by using Chromato-pro software.

Figure 4. Detection of the intestinal permeability of tetracycline hydrochloride by HPLC analysis. (A) Oral administration of tetracycline hydrochloride after 30 min. **(B)** Oral administration of the solvent after 30 min. **(C)** Oral administration of tetracycline hydrochloride after 1.5 h. **(D)** Oral administration of the solvent after 1.5 h. **(E)** Time course of the detection of intestinal permeability of tetracycline hydrochloride by HPLC analysis. The peak area ratio was calculated by using Chromato-pro software.

Figure 5. Detection of the intestinal permeability of chloramphenicol by HPLC analysis. (A) Oral administration of chloramphenicol after 30 min. **(B)** Oral administration of the solvent after 30 min. **(C)** Oral administration of chloramphenicol after 1.5 h. **(D)** Oral administration of the solvent after 1.5 h. **(E)** Time course of the detection of intestinal permeability of chloramphenicol by HPLC analysis. The peak area ratio was calculated by using Chromato-pro software.

injected orally into the *B. mori* larvae. Theophylline was injected at 1.25 mg/g body weight of *B. mori* at a volume of 0.18 mL, and the hemolymph was collected after 30 min. We detected the peak that indicates theophylline by HPLC (Figures 3A and 3C, indicated by arrow). Furthermore, the peak confirmed that theophylline was absorbed from the midgut 30 min after administration. The peak reached its maximum intensity at 3 h, and then gradually decreased (Figure 3E).

Similarly, tetracycline hydrochloride was injected at 1.5 mg/g body weight of *B. mori* at a volume of 0.18 mL, and the peak indicating that the tetracycline hydrochloride penetrated the hemolymph from the midgut was detected from 30 min to 9 h after administration (Figures 4A and 4C, indicated by arrow). The peak reached its maximum intensity at 3 h and then decreased (Figure 4E).

Chloramphenicol was injected at 0.6 mg/g body weight of *B. mori* at a volume of 0.18 mL, and the peak indicating that the chloramphenicol penetrated the hemolymph from the midgut was detected from 30 min to 9 h after administration (Figures 5A and 5C, indicated by arrow). Interestingly, chloramphenicol had the largest peak area in the collected hemolymph at 1.5 h after administration (Figure 5C). When the amount of chloramphenicol absorbed from the midgut was examined, the maximum concentration of the drug occurred at 1.5 h after the administration and then gradually decreased (Figure 5E).

Retention times were 23.5 ± 0.17 min (mean $\pm$ SD) for theophylline, 25.7 ± 0.02 min for tetracycline hydrochloride, and 36.4 ± 0.42 min for chloramphenicol. Thus, theophylline, tetracycline hydrochloride, and chloramphenicol permeated through the midgut to the hemolymph 30 min after administration (Figures 3A, 4A, and 5A). No remarkable peak was detected in each control solvent (Figures 3B, 3D, 4B, 4D, 5B, and 5D). Hence, the intestinal permeabilities of theophylline, tetracycline hydrochloride, and chloramphenicol could be evaluated by using the *B. mori* model.

4. Discussion

First, we analyzed the RNA-seq data of the day 3 fifth-instar *B. mori* larval midgut to compare the characteristics between the human small intestine and *B. mori* larval midgut. Interestingly, 16 ABC transporters and 7 SLC transporter genes were commonly expressed in the *B. mori* larval midgut and human intestine. The ABCG transporter genes were well-conserved between *B. mori* and human; however, Caco-2 cells did not express ABCG transporters. Although 11 SLC transporter genes were identified in the *B. mori* larval midgut, SLC transporter genes and their function have not been analyzed in *B. mori*.

Fifty-one ABC transporter genes have been identified in *B. mori* and are categorized into eight classes (*21*). However, the function of most ABC transporter genes has not been analyzed without the *B. mori* ABC transporter subfamily C member 2 (*BmABCC2*), which relays transported Cry toxins from *Bacillus thuringiensis* (*22*). Aditionally, a single amino acid mutation of *BmABCC2* causes a resistance to Cry toxin in *B. mori* (23). BmABCC2 has high homology with the human *ABCC4*, also known as multi-drug resistance protein 4, which is involved in organic anion transport and drug resistance. The midgut RNA-seq data showed that *BmABCC2* mRNA expression was greater than that of the other transporters in day 3 fifth-instar *B. mori* larvae; also, human *ABCC4* was expressed in the small intestine in the public RNA-seq data that we employed. Thus, human *ABCC4* and *BmABCC2* might share with similar function.

While the dietary amount increases from day 3 to day 5 in fifth-instar *B. mori* larvae in *B. mori*, their body size is also increases from day 3 to day 5 (*24*). Thus, in this study, day 3 fifth-instar *B. mori* larvae would be appropriate for evaluation of the intestinal permeability of compounds. It is suggested that the functions of the *B. mori* larval midgut and the human small intestine are similar in terms of intestinal permeability of compounds.

Next, we developed an oral administration method for day 3 fifth-instar *B. mori* larvae without damaging the oral cavity and gastrointestinal tract. In addition, we used a relatively large *B. mori* strain, Kinshu × Showa, which remained fasting for 24 h, so we could easily inject *via* oral administration. We could thus determine the dose per weight.

Then, we used *B. mori* larvae to examine the intestinal absorption of three compounds with clear intestinal absorption in humans. Theophylline, tetracycline hydrochloride, and chloramphenicol were examined for their intestinal permeability (*http://www.genome.jp/kegg/drug/*) because these compounds showed clear dose-response curves in *B. mori* larva. In humans, the time of maximum concentration (T_{max}) of theophylline has been reported as 24 h, whereas those of tetracycline hydrochloride and chloramphenicol have been reported as 2 and 3 h, respectively (*http://www.genome.jp/kegg/drug/*). In this study, the T_{max} values of theophylline, tetracycline hydrochloride, and chloramphenicol for *B. mori* correlated with the human T_{max} values.

The LD_{50} values in *B. mori* for tetracycline and chloramphenicol were comparable to those in mice. According to the material safety data sheet (MSDS), the LD_{50} is 0.24 mg/g for theophylline, 2.8 mg/g for tetracycline hydrochloride, and 1.5 mg/g for chloramphenicol with oral administration in mice. However, the LD_{50} for theophylline did not agreed with that in mice data in this study. Thus, LD_{50} does not necessarily need to match with mice data in the transition of the plasma concentration of theophylline

because the transition of the plasma concentration of theophylline in *B. mori* was similar to that in humans. The lethal dose and intestinal permeability can be considered to be different factors in drug characterization. Furthermore, the peaks that indicated the absorption of these compounds from the midgut could be detected by HPLC analysis approximately 30 min after oral administration.

Although theophylline and tetracycline might be substrates of some transporters, such as *SLC22A6*, *SLC22A7*, *SLC22A8*, *SLC22A9* (*25*), it is unclear if these transporters are responsible for elimination of theophylline and tetracycline from the small intestine. *SLC22A6* is related to transport of tetracycline. *SLC22A6* is involved in the excretion of substrates in human. Additionally, *SLC22A6* was highly expressed in *B. mori*, but, it was not expressed in the small intestine of the human. Thus, differences in the expression of drug transporters may cause differences in drug absorption. However, the T_{max} of tetracycline in *B. mori* was consistent with that through the human small intestine. This result indicated that tetracycline could be evaluated by using a *B. mori* model without effects of *SLC22A6*. However, we only examined the expression of human homolog transporters in *B. mori* by using midgut RNA-seq data. In the future studies, it will be necessary to clarify where these transporters are expressed and how they are involved in drug absorption in the midgut cells in *B.mori*.

In addition, the absorption of these compounds might be affected by the peritrophic membrane in *B. mori* larvae. Pesticides with molecular weights of 50-450 have shown oral toxicity in insects (*26*); thus, compounds with molecular weights of ≤ 500 may be easily absorbed from the *B. mori* larval midgut. Using the isolated *B. mori* larval midgut, Hamamoto *et al.* (*12*) reported that the relationship between the polarity and molecular weight of a compound determines their intestinal permeability. In this experiment, we showed that membrane permeation in the midgut was induced by compounds that are more hydrophobic than hydrophilic and that membrane permeation was more likely induced by compounds of smaller molecular weight. Furthermore, Hamamoto *et al.* (*12*) mentioned that the isolated midgut of the *B. mori* model has similar intestinal permeability to those in humans and mice.

The human small intestine has a pH of 5-7 in the upper part and 7-8 in the lower part (*27*), whereas the *B. mori* larval midgut has a pH of approximately 11 (*28*). Differences in pH may cause structural changes in some compounds when it passes through the midgut. For this reason, when administering a compound, we should consider suitable solvents and adjust the pH inside the midgut to be equal to that of the human small intestine.

The method for evaluating the intestinal absorption of a compound used in this study is much simpler than the pre-existing intestinal perfusion system. Furthermore, the prediction of intestinal absorption of a compound *via* transporters would be more appropriate than existing methods because whole bodies were used in this study. As an advantage, *B. mori* can be maintained throughout the year by using an artificial diet, and large numbers of genetically homogeneous individuals can be kept in a narrow space at low cost. In addition, there are no animal ethics problems.

Prediction of the intestinal permeability of some compounds in humans is difficult because of species differences and bioavailability. If we can produce transgenic silkworms that express human drug transporters in the midgut, we will be able to solve this problem; therefore, *B. mori* larva is considered helpful for evaluation of the intestinal permeability of a compound at the screening stage. In sum, we propose the use of *B. mori* larvae to evaluate intestinal absorption of compounds. This system will be useful for evaluation of intestinal permeability in medical drug development.

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