Establishment of systematic approach for selection of prodrugs designed to improve oral absorption

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July 2020

Dissertation

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ABBREVIATIONS

ADME	Absorption, distribution, metabolism, and excretion
BNPP	Bis-*p*-nitrophenyl phosphate
BSA	Bovine serum albumin
CES	Carboxylesterase
$C_{equilibrium}$	Equilibrium concentration
CL_{int}	Intrinsic clearance
$CL_{int,liver}$	CL_{int} in liver
$CL_{int,serum}$	CL_{int} in serum
$CL_{int,small\ intestine}$	CL_{int} in small intestine
$CL_{int,total}$	The sum of $CL_{int,small\ intestine}$, $CL_{int,liver}$, and $CL_{int,serum}$
CR	Conversion ratio
CR_{liver}	CR in liver
CR_{serum}	CR in serum
$CR_{small\ intestine}$	CR in small intestine
CS	Conversion score
CS_{liver}	CS in liver
CS_{serum}	CS in serum
$CS_{small\ intestine}$	CS in small intestine
CS_{total}	The sum of $CS_{small\ intestine}$, CS_{liver}, and CS_{serum}
DMSO	Dimethyl sulfoxide
F	Bioavailability
FaSSIF	Fasted-state simulated intestinal fluid
FeSSIF	Fed-state simulated intestinal fluid
F_g	First pass effect in gut
HBSS	Hanks' balanced salt solution
JP1	Japanese pharmacopoeia 1st fluid

JP2	Japanese pharmacopoeia 2nd fluid
LC/MS	Liquid chromatography-mass spectrometry
LC-MS/MS	Liquid chromatography-tandem mass spectrometry.
NADPH	Nicotinamide adenine dinucleotide phosphate
P_{app}	Apparent permeability coefficients
P_{app_AM}	P_{app} value of active metabolite
P_{app_PD}	P_{app} value of prodrug
$P_{app\_PD\ total}$	The sum of P_{app_PD} value and $P_{app\_PD>AM}$
$P_{app\_PD>AM}$	P_{app} value of active metabolite produced
PBS	Phosphate-buffered saline
P_e	Permeability coefficients
PK	Pharmacokinetic
R	Membrane retention

GENERAL INTRODUCTION

In drug development, we sometimes encounter disadvantages of new chemical entities such as low bioavailability, short duration of action, and toxicity. One of the effective strategies to overcome them is the development of prodrugs (Rautio et al., 2008; Huttunen et al., 2011; Akhani et al., 2017). Prodrugs, which are pharmacologically inactive forms, are subjected to enzymatic or non-enzymatic biotransformation to form active metabolites in the body. Many prodrugs have been developed by the improvement of membrane permeability or solubility, because gastrointestinal permeability and solubility of drugs are the determinants to control the absorption and bioavailability of drugs. For example, enalapril, oseltamivir, and temocapril were developed to improve oral absorption by increasing of the permeability of the pharmacologically active compounds, whereas estramustine phosphate, fosamprenavir, and prednisolone phosphate were those by increasing the solubility of the pharmacologically active compounds (Rautio et al., 2008).

Although the concept of the some developed prodrugs on sale has been publicly disclosed, there is limited information on the process for the development of efficient prodrug (Rautio et al., 2008; Huttunen et al., 2011; Akhani et al., 2017). Design for prodrugs has been well discussed focusing on certain active compounds (Nofsinger et al., 2014; Schade et al., 2014), but general systematic strategies to select candidate prodrugs with ideal pharmacokinetics have not matured yet (Jana et al., 2010; Hoppe et al., 2014). In the case of drugs that are pharmacologically active themselves (non-prodrugs), their physicochemical parameters such as log P, molecular weight, and polar surface areas, have been often reviewed for their effects on pharmacokinetic parameters (Veber et al., 2002; Meanwell et al., 2011), whereas those of the marketed prodrugs have not been discussed. As the concept of drug-likeness has been used for optimizing pharmacokinetic properties of marketed drugs (Leeson and Springthorpe, 2007), it should be possible to use the concept of prodrug-likeness to optimize pharmacokinetic properties of candidate prodrugs.

Pre-clinical studies using experimental animals are performed to predict the

pharmacokinetics in humans prior to clinical trials. There are species differences in substrate specificities and tissue distributions of drug-metabolizing enzymes including hydrolases, which are responsible for the conversion of prodrugs into active metabolites in most cases (Cerny, 2016; Fukami and Yokoi, 2012). Thus, similar to non-prodrug, the selection of appropriate experimental animal species is also crucial for prodrug development from the viewpoints of pharmacokinetics and pharmacodynamics.

In Chapter 1, to establish an *in vitro* system for selection of candidate prodrugs and appropriate experimental animals, physicochemical/biopharmaceutical properties (Log D, solubility, and membrane permeability) and metabolic stabilities of 21 selected clinically used prodrugs and their active metabolites were characterized. Twenty-one prodrugs, which had been developed for improved oral absorption of pharmacologically active metabolites, were classified into two types: (1) 16 prodrugs designed to improve membrane permeability of pharmacologically active metabolites and (2) 5 prodrugs designed to improve solubility. By characterizing of physicochemical and metabolic properties of 21 marketed prodrugs and their active metabolites, the purpose of this study is to establish an *in vitro* system for selection of candidate prodrugs and appropriate experimental animals.

In Chapter 2, the utility of the strategy established in Chapter 1 was evaluated by using oseltamivir and 23 oseltamivir analogues, since the used model compounds in Chapter 1 were from clinically used prodrugs (with different structures), and they should meet the criteria for desirable prodrugs. Oseltamivir analogues having various types of side chain were designed as model compounds to have higher log D values than oseltamivir acid, an active metabolite. The physicochemical/biopharmaceutical properties (log D, solubility, and membrane permeability) and metabolic stabilities of these analogues were characterized.

CHAPTER 1

Establishment of systematic approach for selection of prodrugs based on physicochemical/biopharmaceutical properties and metabolic profiles of marketed prodrugs

SUMMARY

To establish a systematic *in vitro* method to appropriately select candidate prodrugs, physicochemical/biopharmaceutical properties of 21 commercially available prodrugs (16 prodrugs with improved membrane permeability of pharmacologically active metabolites and 5 prodrugs with improved solubility) and their active metabolites were characterized in terms of solubility in artificial intestinal fluids and membrane permeability using Caco-2 cells. Their *in vitro* metabolic profiles were also evaluated, using human and animal enterocytes, hepatocytes, and sera. Log D values of prodrugs with improved membrane permeability were higher than those of their active metabolites, whereas those of prodrugs with improved aqueous solubility were lower than those of active metabolites. The prodrugs with improved aqueous solubility were highly soluble in artificial intestinal fluids. All prodrugs were efficiently converted to active metabolites by human matrices, whereas some were not by dog or monkey matrices. This study demonstrated that physicochemical/biopharmaceutical properties could be useful information to facilitate the designing of prodrugs and for selection of candidate prodrugs. Moreover, the *in vitro* evaluation of conversion efficiency to active metabolites would be helpful for selecting ideal prodrugs as well as appropriate animals for *in vivo* PK studies.

INTRODUCTION

Duration and cost required for drug development tend to increase year by year, and the

success rate in development of drugs tends to decrease. For the development of effective and safe drugs, it is required to establish an efficient systematic strategy. Development of prodrug is one of the useful strategies for overcoming various defects of compounds such as low bioavailability, short duration of action, and toxicity (Rautio et al., 2008; Huttunen et al., 2011; Akhani et al., 2017). In fact, approximately 10% of marketed drugs are prodrugs (Rautio et al., 2018).

In this chapter, to establish a systematic strategy for selecting efficient candidate prodrugs, the physicochemical/biopharmaceutical properties and metabolic profiles of 21 marketed prodrugs and their active compounds were characterized. The used model prodrugs were azilsartan medoxomil, bacampicillin, benazepril, candesartan cilexetil, cefuroxime axetil, enalapril, fenofibrate, fesoterodine, lenampicillin, mycophenolate mofetil, olmesartan medoxomil, moexipril, oseltamivir, ramipril, sultamicillin, and temocapril, that had been designed to improve membrane permeability of their pharmacologically active compounds, as well as estramustine phosphate, etoposide phosphate, fosamprenavir, prednisolone phosphate, and tedizolid phosphate, that had been designed to improve solubility of their pharmacologically active compounds.

EXPERIMENTAL PROCEDURES

Chemicals and reagents

Twenty-one marketed prodrugs and their pharmacologically active metabolites were purchased from suppliers shown in Table 1. Japanese pharmacopoeia 1st fluid (JP1) and Japanese pharmacopoeia 2nd fluid (JP2), which are simulated gastric and intestinal fluids, respectively, were purchased from Kanto Chemical Co., Inc., (Tokyo, Japan). The compositions of the buffers are shown in Table 2. Phosphate-buffered saline (PBS) was purchased from Thermo Fisher Scientific Inc. (Waltham, MA). Reagent mixture for simulated intestinal fluid mix was purchased from Celeste (Tokyo, Japan). Bis-p-nitrophenyl phosphate (BNPP) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich Co. LLC.

(St. Louis, MO). All other chemicals were of analytical or the highest quality commercially available. Tissue S9 fractions, cryopreserved enterocytes, cryopreserved hepatocytes, and sera from four species (human, rat, monkey, and dog) shown in Table 3 were purchased from

Table 1. Compounds used in this study.

No.	Prodrug	Manufacturer	Active metabolite	Manufacturer
Prodrugs with improved membrane permeability				
1	Azilsartan medoxomil	A	Azilsartan	B
2	Bacampicillin	C	Ampicillin	B
3	Benazepril	D	Benazeprilat	A
4	Candesartan cilexetil	C	Candesartan	A
5	Cefuroxime axetil	A	Cefuroxime	E
6	Enalapril	C	Enalaprilat	B
7	Fenofibrate	C	Fenofibric acid	D
8	Fesoterodine	A	5-Hydroxymethyl tolterodine	A
9	Lenampicillin	F	Ampicillin	--
10	Mycophenolate mofetil	B	Mycophenolic acid	C
11	Olmesartan medoxomil	E	Olmesartan	G
12	Moexipril	A	Moexiprilat	H
13	Oseltamivir	C	Oseltamivir acid	A
14	Ramipril	C	Ramiprilat	H
15	Sultamicillin	I	Ampicillin and Sulbactam	--
16	Temocapril	E	Temocaprilat	A
Prodrugs with improved aqueous solubility				
17	Estramustine phosphate	J	Estramustine	E
18	Etoposide phosphate	E	Etoposide	C
19	Fosamprenavir	A	Amprenavir	B
20	Prednisolone phosphate	E	Predonisolone	C
21	Tedizolid phosphate	F	Tedizolid	A

--: Duplication or not applicable

A: Toronto Research Chemicals (Toronto, Canada), B: Sigma-Aldrich Co. LLC. (St. Louis, MO), C: FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan), D: Combi-Blocks Inc. (San Diego, CA), E: LKT Laboratories, Inc. (St Paul, MN), F: Haoyuan Chemexpress Co., Ltd (Shanghai, China), G: Tocris Bioscience (Bristol, UK), H: Santa Cruz Biotechnology, Inc. (Dallas, TX), I: AK Scientific, Inc. (Union City, CA), J: MedChemExpress LLC (Princeton, NJ)

Sekisui XenoTech, LLC (Kansas City, KS), In Vitro ADMET Laboratories, Inc. (Columbia, MD), BioIVT (Westbury, NY), and Biopredic International (Rennes, France), respectively.

Table 2. Composition of JP1, JP2, FaSSIF, FeSSIF, and PBS.

	JP1	JP2	FaSSIF	FeSSIF	PBS
pH	1.2	6.8	6.5	5.0	7.0 - 7.3
NaCl (mM)	34.2	--	--	--	136.9
HCl (mM)	82.3	--	--	--	--
KH_2PO_4 (mM)	--	12.5	28.7	--	1.5
Na_2HPO_4 (mM)	--	12.7	--	--	15.5
Taurocholate (mM)	--	--	3.0	15.0	--
Lecithin (mM)	--	--	0.75	3.75	--
Acetic acid (mM)	--	--	--	144.0	--
KCl (mM)	--	--	103.3	203.9	2.7

Determination of log D and solubility of prodrugs and their active metabolites in various buffers

Log D values of the 21 prodrugs and their active metabolites were determined based on the retention time in reversed-phase high-performance liquid chromatography using 7 standard compounds, butyl 4-hydroxybenzoate, ethyl 4-hydroxybenzoate, heptyl paraben, methyl 4-hydroxybenzoate, phenol, propyl 4-hydroxybenzoate, and uracil, as reported previously (Valkó et al., 1997; Du et al., 1998). Solubility of compounds was determined according to methods reported previously with slight modifications (Roy et al., 2001; Alenz and Kansy, 2007). Briefly, each compound was dissolved in DMSO at 10 mM, as a stock solution. Ten µL of the stock solution in 96-well plates were evaporated, and 200 µL of JP1, JP2, fasted-state simulated intestinal fluid (FaSSIF), fed-state simulated intestinal fluid (FeSSIF), or 990 µL of PBS was added and rigorously mixed. The mixtures were centrifuged (5000 rpm, 15°C, 5 min), and the supernatants were subjected to LC/MS (Quatro Premier XE, Waters Corporation, Milford, MA) on a system equipped with an Acquity UPLC BEH C18 column (1.7 µm particle size, 2.1 × 50 mm; Waters Corporation). The mobile phase consisted of (A) 20 mM ammonium acetate/5% acetonitrile and (B) 10 mM ammonium acetate/95%

Table 3. Donor information of various matrices.

Species	Strain, breed, or race	Sex (animals or individuals)	Age	Lot No.
Human				
Small intestine S9 fractions	AA (2) and C (4)	Pool of 3 M and 3 F	15 – 66 years	1410064
Liver S9 fractions	AA (9), A (3), C (172), and H (16)	Pool of 100 M and 100 F	11 - 83 years	0910256
Cryopreserved enterocytes	C	1 F	44 years	HE3009
Cryopreserved hepatocytes	C (17), H (2), and O (1)	Pool of 10 M and 10 F	3 Days – 80 years	UYG
Serum	Not available	Pool of 13 M and 13 F	27 - 68 years	SER019A050C001
Rat				
Small intestine S9 fractions	Sprague-Dawley	Pool of 200 M	~ 8 weeks	1410151
Liver S9 fractions	Sprague-Dawley	Pool of 207 M	~ 7 weeks	0610331
Cryopreserved enterocytes	Sprague-Dawley	Pool of 6 M	8 - 12 weeks	RSE3023
Cryopreserved hepatocytes	Sprague-Dawley	Pool of 30 M	10-11 weeks	MSO
Serum	Sprague-Dawley	Pool of 70 M and 35 F	10 – 19 weeks	5H1552
Cynomolgus monkey				
Small intestine S9 fractions	--	1 M	3 years	0710573
Liver S9 fractions	--	Pool of 4 M	5 – 6 years	0210315
Cryopreserved enterocytes	--	1 M	27 months	PCE3012
Cryopreserved hepatocytes	--	Pool of 3 M	8 - 13 years	HEL
Serum	--	Pool of 21 M and 13 F	20 months – 74 months	5K2003
Dog				
Small intestine S9 fractions	Beagle	Pool of 3 M	> 6 months	1410280
Liver S9 fractions	Beagle	Pool of 8 M	0.5 – 3 years	0810316
Cryopreserved enterocytes	Beagle	1 M	3 – 4 months	DBE3026
Cryopreserved hepatocytes	Beagle	Pool of 3 M	6 months	SCD
Serum	Beagle	Pool of 10 M and 5 F	3 – 6 years	6G1423

AA: African American, C: Caucasian, A: Asian, H: Hispanic, O: Oriental, M: male, F: female, (): Number of donors.

--: Not applicable.

acetonitrile. The flow rate was 0.8 mL/min, and the gradient conditions for elution were as follows: gradient [min, B%] = [0, 0] - [1.0, 100] - [1.5, 100] - [1.7, 0]. Mass spectrometric conditions for individual analytes are shown in Table 4.

Membrane permeability of prodrugs and their active metabolites

Membrane permeability of the 21 marketed prodrugs and their active metabolites was determined using Caco-2 cells monolayers according to a method previously reported (Imai et al., 2005; Ohura et al., 2010) in the absence or presence of BNPP, a general inhibitor of serine hydrolases (Oda et al., 2015). Membrane permeability of propranolol was also evaluated as a marker compound with high permeability. Transport buffer in apical side was Hanks' balanced salt solution (HBSS) containing 25 mM D-(+)-glucose and 20 mM 2-(*N*-morpholino) ethanesulfonic acid (pH 6.5), whereas that in basolateral side was HBSS containing 25 mM D-(+)-glucose, 25 mM 4-(2-hydoxyethyl)-1-piperazineethanesulfonic acid, and 4.5% BSA (pH 7.4). BNPP dissolved in the transport buffer (pH 6.5) was added to both apical and basolateral sides at a final concentration of 200 μM. After 20 min of incubation, prodrugs or their active metabolites were added to the apical side at 25 μM, and incubated at 37˚C for 2 h. The compounds transported to basolateral side were measured using an LC-MS/MS system (Xevo TQS, Waters Corporation) equipped with an Acquity UPLC BEH C18 column (1.7 μm particle size, 2.1 × 50 mm; Waters Corporation). The mobile phase consisted of (A) 0.1% formic acid and (B) 0.1% formic acid/acetonitrile. The flow rate was 1.0 mL/min, and the gradient condition was as follows: gradient [min, B%] = [0, 2] - [0.2, 2] - [0.4, 98] - [1.0, 2]. Mass spectrometric conditions for individual analytes are shown in Table 4. When prodrugs were added to the apical side, the concentrations of their active metabolites in basolateral side

Table 4. LC-MS/MS or LC-MS conditions for compounds.

No.	Prodrug	Electrospray ionization	m/z	Collision energy	Active metabolite	Electrospray ionization	m/z	Collision energy
Prodrug with improved membrane permeability								
1	Azilsartan medoxomil	Positive	569.15 > 233.15	40	Azilsartan	Positive	457.17 > 233.13	30
2	Bacampicillin	Positive	466.08 > 276.09	10	Ampicillin	Positive	350.10 > 105.98	20
3	Benazepril	Positive	425.19 > 351.11	20	Benazeprilat	Positive	397.20 > 351.09	20
4	Candesartan cilexetil	Positive	611.23 > 423.01	10	Candesartan	Positive	441.15 > 263.13	10
5	Cefuroxime axetil	Positive	511.13 > 458.94	10	Cefuroxime	Positive	425.07 > 270.97	20
6	Enalapril	Positive	377.21 > 117.02	40	Enalaprilat	Positive	349.16 > 116.97	30
7	Fenofibrate	Positive	361.07 > 138.93	30	Fenofibric acid	Positive	319.01 > 233.05	20
8	Fesoterodine	Positive	412.07 > 222.97	40	5-Hydroxymethyl tolterodine	Positive	342.14 > 223.11	30
9	Lenampicillin	Positive	462.15 > 160.05	20	Ampicillin	Positive	350.10 > 105.98	20
10	Mycophenolate mofetil	Positive	434.02 > 114.02	40	Mycophenolic acid	Positive	321.02 > 207.10	20
11	Olmesartan medoxomil	Positive	559.32 > 207.09	30	Olmesartan	Positive	447.18 > 207.08	30
12	Moexipril	Positive	499.29 > 234.15	20	Moexiprilat	Positive	471.23 > 206.12	20
13	Oseltamivir	Positive	313.65 > 166.07	20	Oseltamivir acid	Positive	285.11 > 197.08	10
14	Ramipril	Positive	417.23 > 117.01	40	Ramiprilat	Positive	389.10 > 206.14	20
15	Sultamicillin	Positive	595.14 > 113.97	40	Ampicillin	Positive	350.10 > 105.98	20
16	Temocapril	Positive	477.00 > 270.04	20	Temocaprilat	Positive	449.07 > 270.03	20
Prodrug with improved aqueous solubility								
17	Estramustine phosphate	Negative	518.13	--	Estramustine	Positive	440.18	--
18	Etoposide phosphate	Positive	669.16	--	Etoposide	Positive	589.19	--
19	Fosamprenavir	Positive	586.20	--	Amprenavir	Positive	506.23	--
20	Prednisolone phosphate	Negative	441.17	--	Predonisolone	Positive	361.20	--
21	Tedizolid phosphate	Positive	451.09	--	Tedizolid	Positive	371.13	--

--: Not applicable

were also measured, because prodrugs can possibly decompose spontaneously or be hydrolyzed in Caco-2 cells or by added BSA. Apparent permeability coefficients (P_{app}) were calculated as follows:

Q (nmol) = Measured concentration (nmol/L) × volume of basolateral side (0.001 L)

P_{app} (cm/s) = (dQ/dt) / (A × C_0)

where dQ/dt is apparent transport rate (nmol/s), "t" represents the incubation time (7200 s), "A" represents the membrane surface area (0.3 cm^2/well), and "C_0" represents the nominal concentration before incubation (25 μM). P_{app} values of prodrugs ($P_{app\_PD\ total}$) were the sum of P_{app} values of prodrugs themselves (P_{app_PD}) and P_{app} values of the active metabolites ($P_{app\_PD>AM}$) produced, which were calculated using Q values of active metabolite and C_0 value of nominal concentration of prodrug before incubation. As for the evaluation of permeability of active metabolites, P_{app} values (P_{app_AM}) were calculated based on the concentration of each active metabolite in basolateral side.

Evaluation of metabolic stability of prodrugs and their active metabolites

To determine the stability of the prodrugs, the conversion of prodrugs to active metabolites was evaluated using small intestine and liver S9 fractions, cryopreserved enterocytes, cryopreserved hepatocytes, and sera from humans and experimental animals. Prodrugs were dissolved in acetonitrile/DMSO (95:5, v/v) at 500 μM, and were added to the reaction mixture to be a final concentration of 5 μM. The stabilities of prodrugs in small intestine and liver S9 fractions and in sera were determined as follows: a typical incubation mixture (final volume: 0.2 mL) contained 100 mM potassium phosphate buffer (pH 7.4), a nicotinamide adenine dinucleotide phosphate (NADPH)-generating system, and enzyme sources [10% (serum) or 0.2 mg/mL (S9 fractions)]. The mixture was incubated for 0, 5, and 30 min at 37°C. The reactions were terminated by the addition of 200 μL of acetonitrile. The stabilities of prodrugs in enterocytes and hepatocytes were determined as follows: a typical

incubation mixture (final volume of 0.1 mL) contained Williams' E Medium (pH 7.4) and enterocytes or HBSS (pH 7.4, in the presence or absence of 10% serum from each species) and hepatocytes. The mixture (cell density: 0.2×10^6 cells/mL) was incubated for 0, 10, 60, and 120 min at 37°C in a CO_2 incubator. The reactions were terminated by the addition of 100 µL of acetonitrile. The mixtures were centrifuged at 3000 rpm, 15°C for 20 min to collect the supernatants. The concentrations of prodrugs or their active metabolites in the sample were measured.

Next, the metabolic instability of active metabolites was examined using small intestine and liver S9 fractions, cryopreserved enterocytes, cryopreserved hepatocytes, and sera from humans and experimental animals. A typical incubation mixture was the same as those for prodrugs except for the cell density of enterocytes and hepatocytes at 0.5×10^6 cells/mL. The mixtures containing small intestine and liver S9 fractions or serum were incubated for 0, 10, and 60 min at 37°C. The mixtures containing enterocytes or hepatocytes were incubated for 0, 60, 120, and 240 min at 37°C in a CO_2 incubator. Subsequent experimental procedures were similar to those for the prodrugs.

The concentrations of prodrugs or active metabolites in the samples were measured using an LC/MS system equipped with SQ detector or TQ detector (Waters Corporation), and an Acquity UPLC BEH C18 column (1.7 µm particle size, 2.1 × 50 mm; Waters Corporation). The mobile phase consisted of (A) 0.1% formic acid and (B) 0.1% formic acid/ acetonitrile. The flow rate was 0.5 mL/min, and the gradient conditions for elution were as follows: gradient [min, B%] = [0, 2] - [1.8, 98] - [2.5, 2] - [2.8, 2].

In vitro metabolic stabilities of prodrugs or their active metabolites were evaluated by calculating the remaining amounts after the incubation. Half-life and intrinsic clearance (CL_{int}) were calculated using the following equations, as reported previously (Obach et al., 1997):

$CL_{int} = 0.693 \times [1 / t_{1/2}\ (min)]$

$\times$ [1/ concentration of matrix (mg/mL or 10^6 cells/mL)]

$\times$ [scale-up factor (mg/g or 10^6 cells/g)]

$\times$ [tissue weight (g) / body weight (kg)]

The *in vitro* CL_{int} in cryopreserved enterocytes, cryopreserved hepatocytes, small intestine and liver S9 fractions, and sera from humans and experimental animals was scaled to the *in vivo* CL_{int} using scaling factors shown in Table 5.

Table 5. Physiological constants and scaling factors in human and animals.

	Human	Rat	Monkey	Dog
Scaling factor				
Small intestine S9 fractions (mg/g)	18	18	18	18
Liver S9 fractions (mg/g)	80.7	87	80.7	80.7
Cryopreserved enterocytes (million cells/g)	539	539	539	539
Cryopreserved hepatocytes (million cells/g)	120	120	120	120
Tissue weight				
Small intestine (g)	2100	11	230	1.5
Liver (g)	1799	9.15	124	1.3
Plasma (g)	3000	9	220	1
Body weight (kg)	70	0.25	5	0.022

The values of scaling factors except for liver S9 fractions in rat were assumed to be similar to humans and the same values were used. Scaling factors of small intestine and liver S9 fractions were assumed to be similar to cytosol fractions (Strikwold et al., 2013). Since there was no report about scaling factor of cryopreserved enterocytes, it was set on the assumption that the area of the small intestine was 200 m^2 and the cell size was 15 μm. Scaling factor of cryopreserved hepatocytes was reported in Bayliss et al (1999).

Calculation of conversion ratio (CR) and conversion score (CS)

To estimate the contribution of hydrolysis to overall metabolism of the prodrugs, two novel parameters were set. The ratio of the amount of active metabolite to amount of all metabolites after an in vitro metabolic instability using each matrix was defined as conversion ratio (CR), as follows:

CR = [Formation of active metabolite (%)] / {100 – [Remaining prodrug (%)]}

$CR_{small\ intestine}$, CR_{liver}, or CR_{serum} represent the rate of conversion in each tissue. The sum of $CL_{int,small\ intestine}$, $CL_{int,liver}$, and $CL_{int,serum}$ was defined as $CL_{int,total}$, representing the intrinsic clearance in body. To estimate the conversion efficacy in each tissue, conversion score (CS) was defined. $CS_{small\ intestine}$, CS_{liver}, or CS_{serum} represent the ratio of the intrinsic clearance of prodrug that was converted to active metabolite in each tissue to the that in body, and was calculated using the following equations:

$$CS_{small\ intestine} = CR_{small\ intestine} \times CL_{int,small\ intestine} / CL_{int,total}$$

$$CS_{liver} = CR_{liver} \times CL_{int,liver} / CL_{int,total}$$

$$CS_{serum} = CR_{serum} \times CL_{int,serum} / CL_{int,total}$$

The sum of $CS_{small\ intestine}$, CS_{liver}, and CS_{serum} was defined as CS_{total}, and CS_{total} should ideally be 1.0, if prodrugs were efficiently converted to active metabolites in human body.

RESULTS

Log D values of prodrugs and their active metabolites

Twenty-one prodrugs (Table 1), which had been developed for improved oral absorption of pharmacologically active metabolites, were classified into two types: (1) 16 prodrugs designed to improve membrane permeability of pharmacologically active metabolites and (2) 5 prodrugs designed to improve solubility. To clarify physicochemical properties of prodrugs and their active metabolites, their log D values were determined. Figure 1 shows the relationship between the log D values of prodrugs and those of their active metabolites. For the 16 prodrugs with improved membrane permeability, the log D values were 1.7 to 4.2 times higher than those of their active metabolites. On the other hand, for the 5 prodrugs with improved aqueous solubility, the log D values were 0.4 to 0.6 times less than

those of their active metabolites. Thus, it was demonstrated that the lipophilicity of the prodrugs had been changed from that of their active metabolites in accordance with the intention of their design.

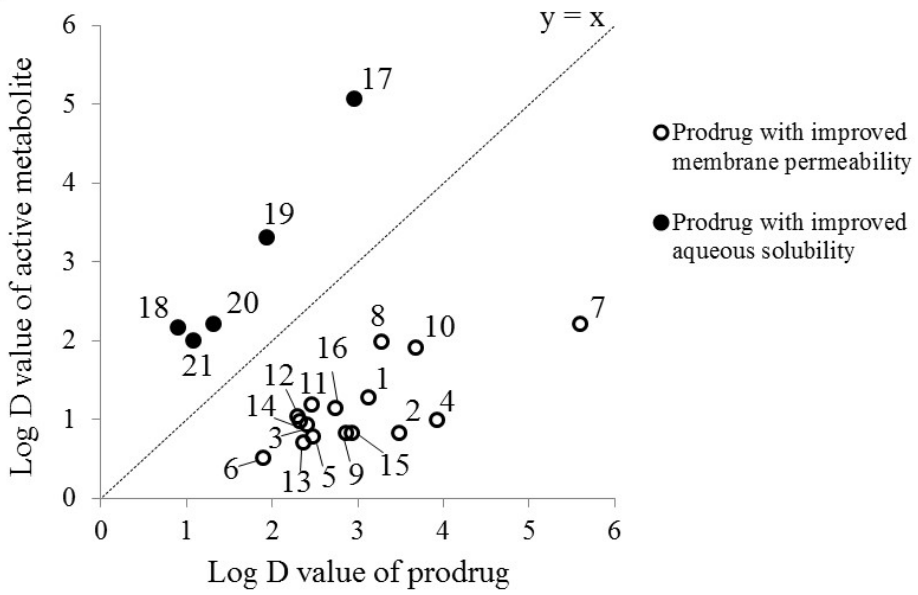


Fig. 1. Relationship between log D values of prodrugs and those of their active metabolites. Open circles represent 16 prodrugs with improved membrane permeability and closed circles represent 5 prodrugs with improved aqueous solubility. The numbers of compounds are shown in Table 1.

Solubility of prodrugs and their active metabolites in artificial intestinal fluids

To further investigate physicochemical properties of the 21 prodrugs and their active metabolites, the solubility of each in JP1, JP2, FaSSIF, FeSSIF, or PBS was determined (Table 6). Among 16 prodrugs with improved membrane permeability, the solubilities of ampicillin, enalapril, and fenofibrate could not be evaluated, because these compounds disappeared in the process of evaporation for some reasons. Also, the solubility of sultamicillin could not be evaluated, because it was unstable in JP2, FaSSIF, and PBS. In JP1, benazepril and mycophenolate mofetil showed higher solubility than that of their active metabolite (Fig. 2). In JP2, azilsartan medoxomil, candesartan cilexetil, and mycophenolate mofetil showed lower solubility than that of their active metabolite. The results might be related to their higher lipophilicity than their active metabolites. Similar phenomena were

Table 6. Solubilities (%) of prodrug with improved membrane permeability or aqueous solubility.

Prodrug	JP1		JP2		FaSSIF (%)		FeSSIF		PBS	
	Prodrug	Active metabolite	Prodrug	Active metabolite	Prodrug	Active metabolite	Prodrug	Active metabolite	Prodrug	Active metabolite
Prodrug with improved membrane permeability										
Azilsartan medoxomil	3	2	8	100	13	100	15	100	72	100
Bacampicillin	93	N.E.	70	N.E.	83	N.E.	98	N.E.	100	N.E.
Benazepril	100	37	100	100	100	100	100	100	100	100
Candesartan cilexetil	0	5	2	92	46	92	63	88	81	100
Cefuroxime axetil	94	53	89	99	95	95	98	100	100	81
Enalapril	N.E.	50	N.E.	100	N.E.	100	N.E.	100	N.E.	100
Fenofibrate	N.E.	0	N.E.	100	N.E.	100	N.E.	98	100	100
Fesoterodine	100	100	99	100	96	100	96	97	100	100
Lenampicillin	100	N.E.	84	N.E.	94	N.E.	100	N.E.	100	N.E.
Mycophenolate mofetil	98	17	32	98	91	96	98	93	93	100
Olmesartan medoxomil	96	100	92	99	95	100	92	96	100	100
Moexipril	97	100	100	100	98	100	98	100	100	100
Oseltamivir	97	100	100	99	89	96	95	98	100	100
Ramipril	100	100	100	100	100	99	100	100	100	95
Sultamicillin	80	N.E.	N.E.	N.E.	N.E.	N.E.	56	N.E.	N.E.	N.E.
Temocapril	100	100	100	100	100	100	100	100	100	100
Prodrug with improved aqueous solubility										
Estramustine phosphate	1	1	100	1	100	52	100	44	97	3
Etoposide phosphate	92	92	100	100	100	100	100	100	100	96
Fosamprenavir	100	100	100	72	100	88	97	98	90	86
Prednisolone phosphate	100	100	100	99	100	100	100	100	100	100
Tedizolid phosphate	3	16	100	3	100	5	100	13	100	45

N.E.: Not evaluated. Since ampicillin, enalapril, and fenofibrate are volatile, their solubilities could not be evaluated. In addition, because sultamaicillin was unstable in JP2, FaSSIF, and PBS, data were not obtained.

observed in FaSSIF (azilsartan medoxomil and candesartan cilexetil), FeSSIF (azilsartan medoxomil and candesartan cilexetil), and PBS (azilsartan medoxomil). The other tested prodrugs and their active metabolites showed solubility of over 80%. In the 5 prodrugs with improved aqueous solubility, estramustine phosphate and tedizolid phosphate showed lower solubility in JP1 than that of their active metabolite, although they showed higher solubility in the other buffers. Thus, it was revealed that the solubility of these prodrugs was improved further than that of their active metabolites. The artificial intestinal fluid would be suitable to evaluate whether the solubility of candidate prodrugs is improved compared with their active metabolites.

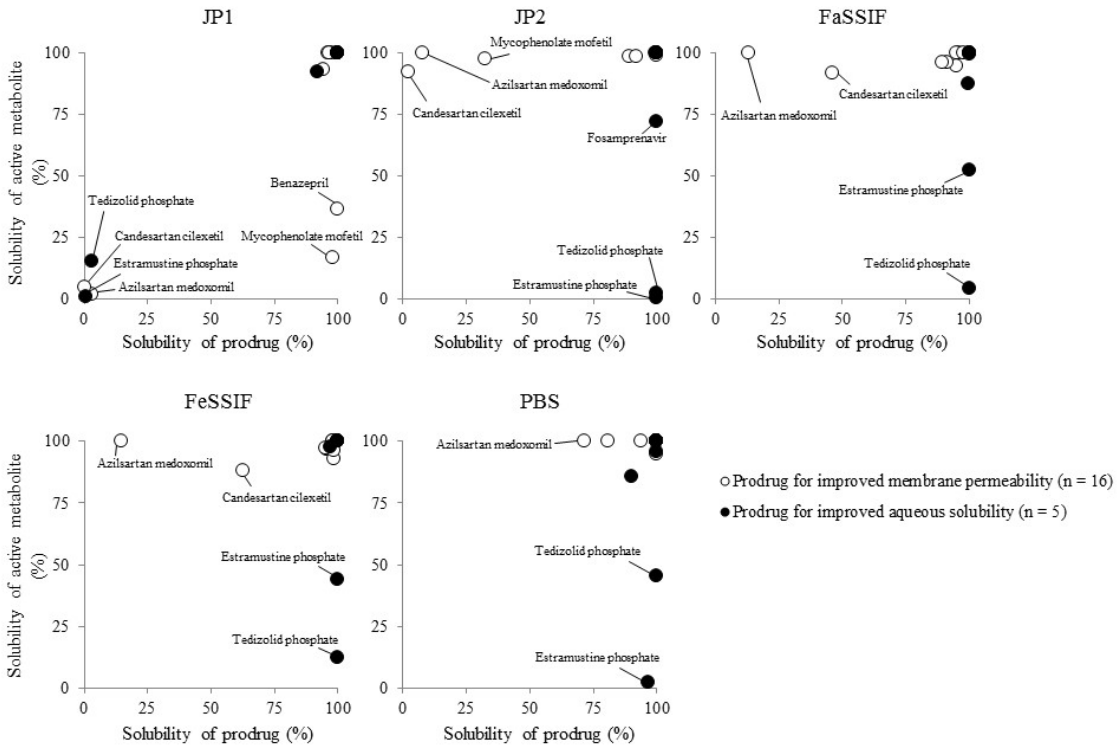


Fig. 2. Solubility of prodrugs and their active metabolites in JP1, JP2, FaSSIF, FeSSIF, and PBS. Open circles represent 16 prodrugs with improved membrane permeability and their active metabolites. Closed circles represent 5 prodrugs with improved aqueous solubility and their active metabolites.

Membrane permeability of prodrugs

To evaluate whether the membrane permeability of each of the prodrugs with improved membrane permeability is higher than that of their active metabolites, apparent permeability

coefficients (P_{app}) of the prodrugs and their active metabolites were evaluated using Caco-2 cells (Table 7). The P_{app} values of cefuroxime axetil, sultamicillin, and cefuroxime could not be calculated, because these compounds were not detected in basolateral side. Of the tested 16 prodrugs, 9 showed higher (more than four times) $P_{app\_PD\ total}$ values than P_{app_AM} values, although the $P_{app\_PD\ total}$ values of enalapril, moexipril, and oseltamivir were almost equal with P_{app_AM} values, and $P_{app\_PD\ total}$ values of fenofibrate and mycophenolate mofetil were more than 20 times lower than their P_{app_AM} values. Moreover, 10 out of 16 prodrugs showed higher $P_{app\_PD>AM}$ values than their P_{app_PD} values, suggesting that these compounds would be efficiently hydrolyzed to their active metabolites in Caco-2 cells or transport buffer in apical side, because carboxylesterase 1 (CES1) catalyzing hydrolysis of various drugs is highly expressed in Caco-2 cells, although it is not expressed in human erythrocytes (Imai et al., 2005). To exclude the factor of the CES1-mediated hydrolysis in Caco-2 cells, a general serine esterase inhibitor BNPP was added. If the active metabolite formed in the cells is transported across the membrane by simple diffusion, it would be equally transported into apical and basolateral sides and taken up again into the cells by the pH-partition hypothesis. Basic compounds are preferentially transferred into apical side (lower pH), whereas acidic compounds are more likely transferred into basolateral side (higher pH). It was expected that $P_{app\_PD\ total}$ and P_{app_PD} values would be increased by the addition of BNPP, if intercellular hydrolysis is decreased, and the amount of active metabolite transferred to the basolateral side is decreased, in the case of neutral or acidic compounds. As shown in Table 4, by the addition of BNPP, the $P_{app\_PD>AM}$ values of benazepril, ramipril, and temocapril were decreased by over one third, and their P_{app_PD} values were increased by 3-fold. However, their $P_{app\_PD\ total}$ values were not largely changed. The $P_{app\_PD>AM}$ values of the other tested prodrugs were not substantially decreased by the addition of BNPP, although the conditions used for BNPP (200 μM) have been reported to sufficiently inhibit hydrolase activity (86% inhibition for in cellulo *O*-butyryl-propranolol hydrolysis) (Ohura et al., 2010). Thus, these prodrugs may have been hydrolyzed by an enzyme(s) that is not inhibited by BNPP, or the hydrolysis reaction might have proceeded during the 2 h incubation by the partially remaining active enzyme.

Table 7. P_{app} ($\times 10^{-6}$ cm/s) values of prodrugs with improved membrane permeability and their active metabolites.

Added compound	BNPP (-)				BNPP (+)			
	Prodrug			Active metabolite	Prodrug			Active metabolite
Measured compound	Prodrug (P_{app_PD})	Active metabolite ($P_{app\_PD>AM}$)	Total ($P_{app\_PD\ total}$)	Active metabolite (P_{app_AM})	Prodrug (P_{app_PD})	Active metabolite ($P_{app\_PD>AM}$)	Total ($P_{app\_PD\ total}$)	Active metabolite (P_{app_AM})
Azilsartan medoxomil	0.23	13.36	13.59	0.30	0.27	12.10	12.38	0.25
Bacampicillin	0.26	21.23	21.48	0.08	1.97	18.45	20.42	0.13
Benazepril	0.20	0.63	0.83	0.09	0.73	0.03	0.76	0.10
Candesartan cilexetil	4.66	13.32	17.97	0.07	14.45	6.28	20.73	0.08
Cefuroxime axetil	N.D.	N.D.	N.C.	N.D.	N.D.	N.D.	N.C.	N.D.
Enalapril	0.06	N.D.	0.06	0.06	0.10	N.D.	0.10	0.12
Fenofibrate	0.08	0.69	0.77	17.18	0.31	0.53	0.84	17.27
Fesoterodine	2.08	2.97	5.05	0.94	2.20	1.95	4.15	0.93
Lenampicillin	0.15	6.99	7.15	0.08	0.19	7.75	7.94	0.13
Mycophenolate mofetil	0.74	N.D.	0.74	40.02	4.57	N.D.	4.57	40.70
Olmesartan medoxomil	0.03	0.41	0.44	0.06	0.06	0.38	0.44	0.07
Moexipril	0.06	0.02	0.09	0.07	0.06	N.D.	0.06	0.07
Oseltamivir	0.10	N.D.	0.10	0.10	0.13	N.D.	0.13	0.10
Ramipril	0.10	0.30	0.40	0.09	0.39	0.10	0.49	0.12
Sultamicillin	N.D.	N.D.	N.C.	0.08	N.D.	N.D.	N.C.	0.13
Temocapril	0.18	1.95	2.13	0.05	0.78	0.46	1.24	0.07

N.D.: Not detected, N.C.: Not calculated.

The P_{app_PD} total values of the prodrugs with improved membrane permeability had been expected to be substantially higher than the P_{app_AM} values, because test compounds were selected as "successful examples." However, the P_{app_PD} total values of fenofibrate and mycophenolate mofetil were lower than their P_{app_AM} values. In addition, in both cases, with or without BNPP, total P_{app_PD} total values of benazepril, enalapril, fenofibrate, mycophenolate mofetil, olmesartan medoxomil, moexipril, oseltamivir, and ramipril were much lower than that of $P_{app\ value}$ (13.3×10^{-6} cm/s) of propranolol, which shows a high fraction of the dose was absorbed (*F*a). Thus, it was demonstrated that the prodrugs with improved membrane permeability did not necessarily show P_{app} values that are higher than those of their active metabolites, and were close to the P_{app} values of propranolol when Caco-2 cells were used. Thus, Caco-2 cells may have some limitation for the evaluation of membrane permeability.

Stability of prodrugs in buffers or media

To examine the extent of non-enzymatic degradation of the prodrugs, their stabilities in potassium phosphate buffer, HBSS, and Williams' E Medium were evaluated (Fig. 3). Potassium phosphate buffer (incubation time: 30 min), HBSS (incubation time: 120 min), and Williams' E Medium (incubation time: 120 min) were used for the reaction mixtures of serum and S9 fractions, cryopreserved hepatocytes, and cryopreserved enterocytes, respectively. Azilsartan medoxomil, bacampicillin, cefuroxime axetil, lenampicillin, and olmesartan medoxomil were unstable in Williams' E Medium (0.0 to 25.2%), whereas they were relatively stable in potassium phosphate buffer (73.5 to 98.7%) and HBSS (48.5 to 80.8%). Fenofibrate was unstable in potassium phosphate buffer (64.4%). Sultamicillin was extremely unstable in all buffers and media (0.0 to 4.9%). The other compounds were stable (over 80%) in all buffers and media. From these results, some marketed prodrugs might be non-enzymatically degraded in in vitro studies and the unstable prodrugs are expected to be naturally hydrolyzed in the body.

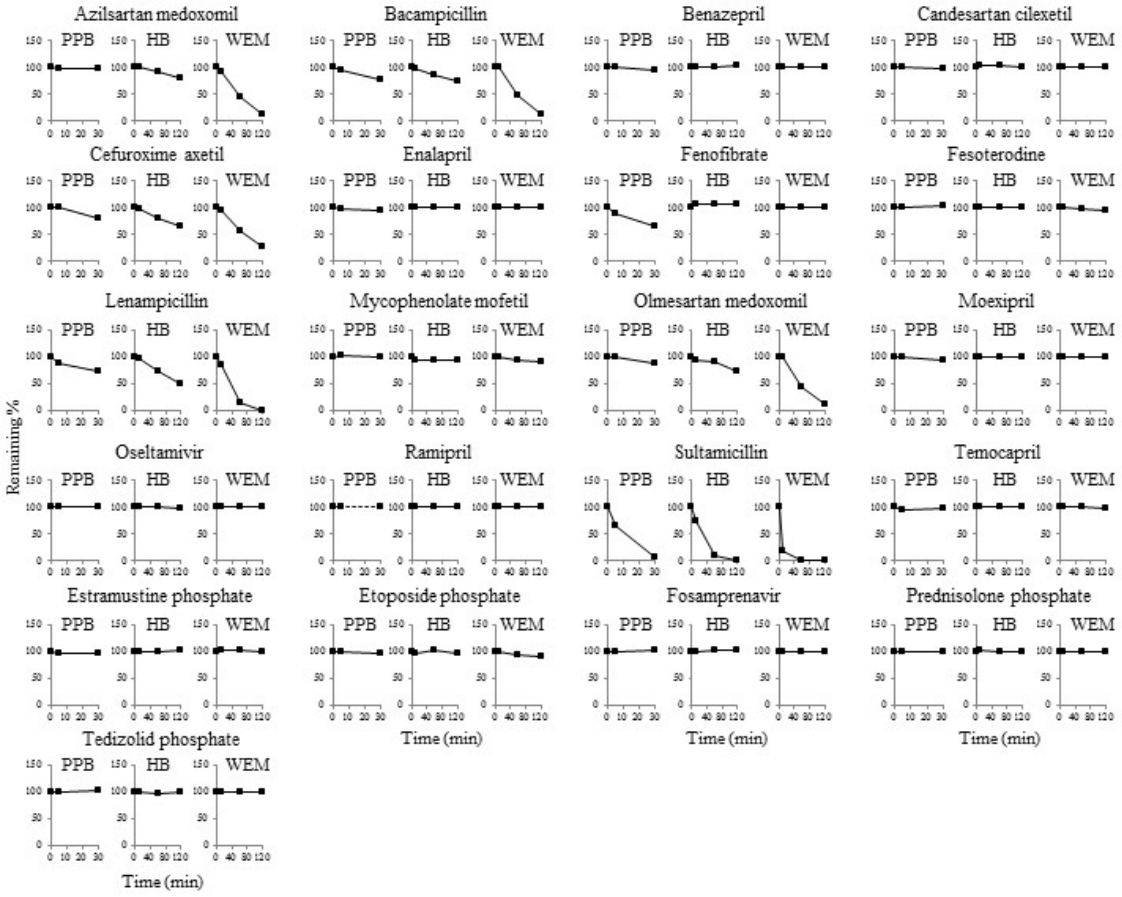


Fig. 3. Stability of prodrugs in buffers or media. Tested prodrugs (5 μM) were incubated with potassium phosphate buffer (PPB), HBSS (HB), or Williams' E Medium (WEM). Closed squares represent the percentages of remaining prodrugs.

Stability and metabolic efficiency of prodrugs by various human and animal matrices

The metabolic stabilities of prodrugs and the formation rate of their active metabolites were determined using human small intestinal and liver S9 fractions, cryopreserved enterocytes, cryopreserved hepatocytes in the presence or absence of human serum, or human serum (Fig. 4). Since there is a lag in the time from the addition of prodrugs to placement of samples into a CO_2 incubator, the samples with cryopreserved enterocytes or hepatocytes at 0 min could contain active metabolites. Prodrugs that were hydrolyzed by all human matrices were azilsartan medoxomil, bacampicillin, cefuroxime axetil, fesoterodine, lenampicillin, mycophenolate mofetil, olmesartan medoxomil, and sultamicillin. As shown in Fig. 3, bacampicillin was unstable in all buffers and media, but the enzymatic hydrolysis appeared to proceed, since the remaining percentages of bacampicillin with all human matrices were

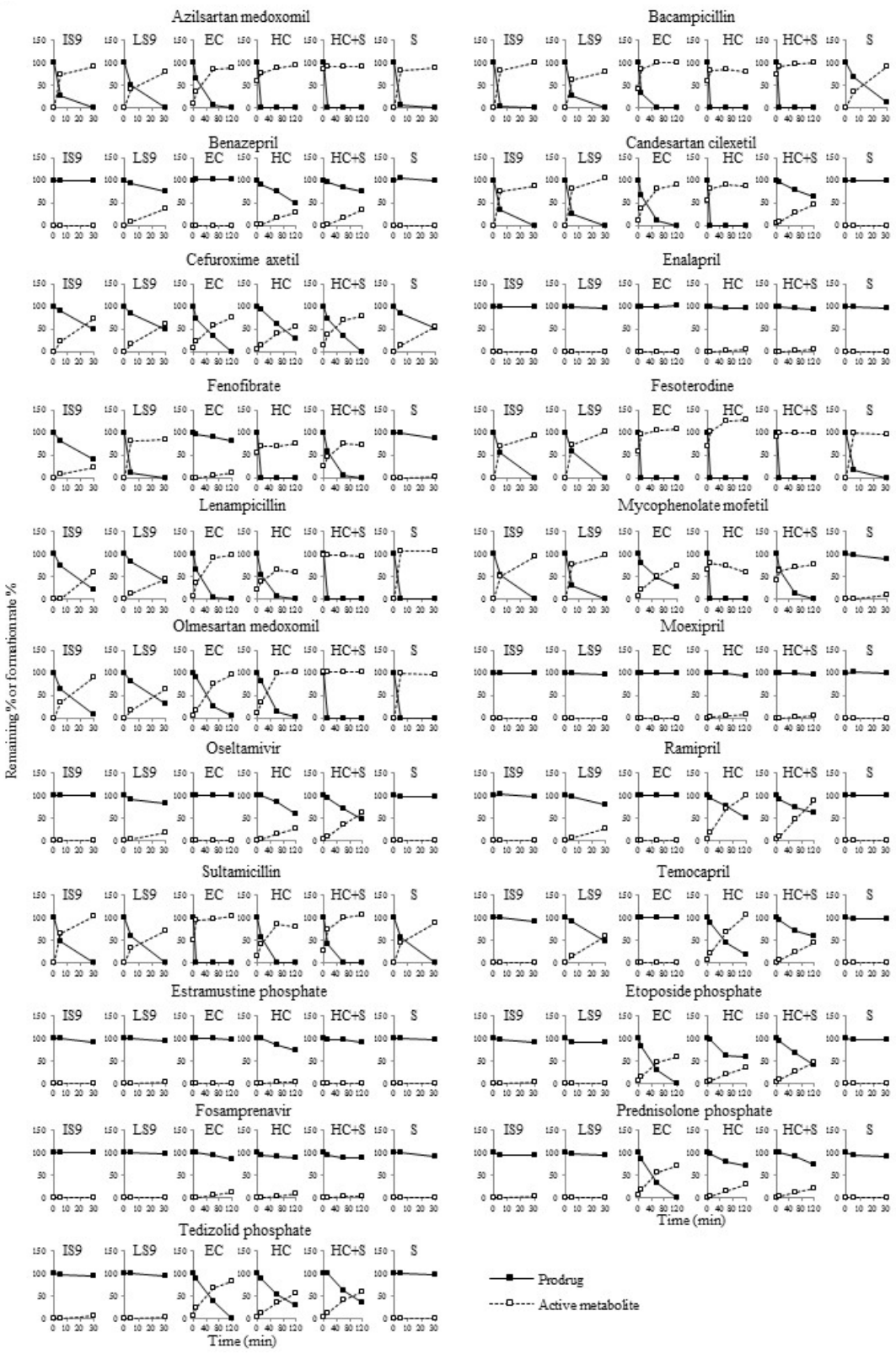


Fig. 4. Stabilities of 21 tested prodrugs in human various matrices. Test prodrugs (5 μM) were incubated with human small intestine S9 fractions (IS9), liver S9 fractions (LS9), enterocytes (EC), hepatocytes in the absence (HC) or presence of serum (HC + S), or serum (S). Closed squares represent the remaining percentages of prodrugs and open squares represent the formation rate of their active metabolites.

smaller than that of spontaneous decomposition. A similar phenomenon was observed in cefuroxime axetil and lenampicillin. Fesoterodine and mycophenolate mofetil were enzymatically hydrolyzed, because they were stable in potassium phosphate buffer, HBSS, and Williams' E Medium. The remaining percentages of sultamicillin with all human matrices were similar to those in all buffers or media; therefore, sultamicillin seems to be non-enzymatically degradated to a great extent. Benazepril, enalapril, fenofibrate, moexipril, oseltamivir, ramipril, and temocapril were hydrolyzed by human liver matrices, consistent with the fact that these prodrugs are hydrolyzed by CES1, which is abundantly expressed in human liver, not in intestine. Candesartan cilexetil and fenofibrate were hydrolyzed by human matrices except for serum. Ishizuka et al. (2013) have reported that candesartan cilexetil is hydrolyzed by hepatic CES1 rather than intestinal enzymes, including CES2, but this study showed that candesartan cilexetil was hydrolyzed in human small intestine S9 fractions as well as in human cryopreserved enterocytes, in which CES1 is not expressed. As for the 5 prodrugs with improved aqueous solubility, estramustine phosphate was slightly hydrolyzed by human cryopreserved hepatocytes in the absence of serum, and the other 4 prodrugs, etoposide phosphate, fosamprenavir, predonisolone phosphate, and tedizolid phosphate were hydrolyzed by human cryopreserved enterocytes and hepatocytes in the presence or absence of serum. Thus, these prodrugs containing phosphoester group were readily hydrolyzed *in cellulo* rather than by tissue S9 fractions. A plausible possibility for this difference might be that the enzyme(s) that are not localized in endoplasmic reticulum or cytosol, or enzymes(s) that require cofactors other than NADPH might be involved in their hydrolysis.

The metabolic stabilities of prodrugs and the formation rate of their active metabolites were also analyzed using matrices from rat, monkey, or dog. The conversion ratio (CR) values in human or animal tissues then were calculated (Table 8). If a prodrug is metabolized to only active metabolites, the CR value should be 1.0. The CR values of several compounds could not be calculated, because of high metabolic stability (over 90%). For most of the prodrugs, CR values in human matrices were over 0.7, indicating that the tested prodrugs are efficiently converted to active metabolites. The apparent clearance of active metabolite formation was

Table 8. Conversion ratio (CR) of prodrug with improved membrane permeability and aqueous solubility in human and animals matrices.

Prodrug	Human						Rat						Monkey						Dog					
	IS9	LS9	EC	HC	HC + S	S	IS9	LS9	EC	HC	HC + S	S	IS9	LS9	EC	HC	HC + S	S	IS9	LS9	EC	HC	HC + S	S
Prodrug with improved membrane permeability																								
Azilsartan medoxomil	1.0	0.8	1.0	1.0	1.0	0.9	1.0	0.8	1.0	1.0	1.0	0.8	0.9	0.9	1.0	1.0	1.0	1.0	1.0	1.0	0.9	0.9	1.0	0.9
Bacampicillin	0.8	0.8	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.9	0.6	0.9	1.0	1.0	1.0	0.6	0.4	1.0	0.7	1.0	1.0	0.6
Benazepril	--	1.0	--	0.6	1.0	--	--	1.0	1.0	1.0	1.0	0.9	0.0	--	--	1.0	0.5	--	--	--	--	0.6	0.2	--
Candesartan cilexetil	1.0	1.0	1.0	1.0	1.0	--	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	--	1.0	1.0	--	1.0	1.0	--
Cefuroxime axetil	1.0	1.0	0.9	1.0	1.0	1.0	1.0	0.7	1.0	1.0	1.0	1.0	1.0	1.0	0.8	0.9	1.0	0.5	1.0	1.0	0.8	0.8	1.0	0.3
Enalapril	--	--	--	--	--	--	--	0.4	--	0.6	1.0	0.6	--	--	--	0.2	--	--	--	--	--	--	--	--
Fenofibrate	0.3	0.9	0.5	1.0	1.0	0.1	0.8	0.7	0.8	1.0	1.0	1.0	0.7	0.9	1.0	1.0	1.0	0.5	0.1	0.9	--	0.4	1.0	0.1
Fesoterodine	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.9	1.0	1.0	1.0	1.0	--	1.0	--	1.0	1.0	1.0	--	1.0	--	0.9	1.0	1.0
Lenampicillin	0.1	0.9	1.0	0.8	1.0	1.0	0.6	0.9	0.5	0.8	1.0	1.0	0.4	1.0	1.0	1.0	1.0	1.0	0.7	0.7	1.0	1.0	1.0	1.0
Mycophenolate mofetil	1.0	1.0	1.0	1.0	1.0	0.9	1.0	0.9	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.6	1.0	1.0	1.0	1.0	1.0	0.8
Olmesartan medoxomil	1.0	0.9	1.0	1.0	1.0	1.0	1.0	0.9	0.9	1.0	1.0	1.0	0.9	0.9	0.9	1.0	1.0	1.0	1.0	1.0	0.9	1.0	1.0	1.0
Moexipril	--	--	--	--	--	--	--	1.0	--	1.0	1.0	1.0	--	--	--	0.3	0.3	--	--	--	--	0.2	0.2	--
Oseltamivir	--	1.0	--	1.0	1.0	--	--	--	--	0.2	1.0	1.0	--	--	--	0.6	0.8	--	--	--	--	1.0	1.0	--
Ramipril	--	1.0	--	1.0	1.0	--	--	1.0	--	1.0	1.0	1.0	--	--	--	1.0	0.6	--	--	--	--	1.0	--	--
Sultamaicillin	1.0	0.8	1.0	0.9	1.0	1.0	1.0	0.7	1.0	1.0	1.0	1.0	0.9	0.8	1.0	0.9	1.0	1.0	1.0	0.9	1.0	1.0	1.0	0.9
Temocapril	--	1.0	--	1.0	0.8	--	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.9	1.0	--	--	1.0	--	1.0	--	--
Prodrug with improved aqueous solubility																								
Estramustine phosphate	--	--	--	0.1	--	--	--	0.1	--	0.1	--	--	--	--	--	0.1	--	--	--	--	--	0.5	--	--
Etoposide phosphate	--	0.0	0.8	0.5	1.0	--	--	--	1.0	1.0	1.0	0.0	--	--	0.5	0.4	0.8	--	--	--	0.2	1.0	0.7	--
Fosamprenavir	--	--	0.8	0.6	0.4	--	--	--	--	1.0	0.7	--	--	--	--	0.4	0.2	0.0	--	--	--	1.0	1.0	--
Prednisolone phosphate	--	--	1.0	0.8	0.8	--	--	--	1.0	0.4	0.8	0.6	--	--	--	0.2	0.9	--	0.0	--	0.7	0.7	0.5	--
Tedizolid phosphate	--	--	1.0	0.9	1.0	--	--	--	1.0	0.6	1.0	0.0	--	--	0.8	0.9	1.0	--	--	--	0.6	0.7	--	1.0

IS9: small intestine S9, LS9: liver S9, EC: cryopreserved enterocytes, HC: cryopreserved hepatocytes, HC + S: cryopreserved hepatocytes with serum, S: serum.

--: Due to high metabolic stability (remaining % of prodrugs were >90%), CR could not be calculated.

calculated by multiplying the value of $CL_{\text{int tissue}}$ by CR values for each tissue ($CR_{\text{small intestine}}$, CR_{liver}, or CR_{serum}). The conversion scores (CSs) for each tissue ($CS_{\text{small intestine}}$, CS_{liver}, or CS_{serum}) were defined as the apparent clearance for active metabolite formation divided by the sum of the $CL_{\text{int,small intestine}}$, $CL_{\text{int,liver}}$, and $CL_{\text{int,serum}}$. The CS_{total}, the sum of $CS_{\text{small intestine}}$, CS_{liver}, and CS_{serum}, represents the ratio of the clearance for active metabolite formation to the total clearance of prodrug. Since prodrug should be converted to the active metabolite in order to exert pharmacological effects, an "effective prodrug" would be completely converted to the active metabolite. The ideal value of CS_{total} for an effective prodrug should be 1.0. CR and CS

Table 9. Conversion score (CS_{total}) of prodrugs with improved membrane permeability and aqueous solubility in human and animals.

Prodrug	Human	Rat	Monkey	Dog
Prodrug with improved membrane permeability				
Azilsartan medoxomil	0.9	0.8	1.0	0.9
Bacampicillin	1.0	0.9	0.9	0.9
Benazepril	1.0	0.9	0.5	0.2
Candesartan cilexetil	1.0	1.0	1.0	1.0
Cefuroxime axetil	1.0	1.0	0.6	0.4
Enalapril	N.C.	0.6	N.C.	N.C.
Fenofibrate	1.0	1.0	1.0	0.5
Fesoterodine	1.0	1.0	1.0	1.0
Lenampicillin	1.0	1.0	1.0	1.0
Mycophenolate mofetil	1.0	1.0	0.9	1.0
Olmesartan medoxomil	1.0	1.0	1.0	1.0
Moexipril	1.0	1.0	0.3	0.0
Oseltamivir	1.0	1.0	0.8	1.0
Ramipril	1.0	1.0	0.7	N.C.
Sultamaicillin	1.0	1.0	1.0	1.0
Temocapril	0.8	1.0	1.0	1.0
Prodrug with improved aqueous solubility				
Estramustine phosphate	N.C.	N.C.	N.C.	N.C.
Etoposide phosphate	1.0	1.0	0.7	0.2
Fosamprenavir	0.8	0.7	0.0	1.0
Prednisolone phosphate	1.0	1.0	1.0	0.7
Tedizolid phosphate	1.0	1.0	1.0	0.7

N.C.: Not calcurated.

values of enalapril, moexipril, and estramustine phosphate could not be calculated, because these compounds were highly stable in human matrices (Table 8). As shown in Table 9, CS_{total} values of the tested prodrugs, except for enalapril, moexipril, and estramustine phosphate, with human matrices were over 0.7, supporting that they were designed to have good metabolic profiles in humans. In rat, CS_{total} values of tested prodrugs were over 0.7 except for enalapril. Enalapril appeared to be hydrolyzed only by rat CESs. In monkey, CS_{total} values for benazepril, cefuroxime axetil, moexipril, and fosamprenavir were below 0.7, and in dog, the values for the former three compounds and for fenofibrate and etoposide phosphate were below 0.7. Thus, monkey may not be always the best animal species for *in vivo* pharmacokinetics studies. CS_{total} values would be useful for the selection of appropriate experimental animals for the *in vivo* PK study of prodrugs.

Stability and metabolic efficiency of active metabolites by various human and animal matrices

To investigate whether the active metabolites are metabolically stable, the remaining percentages of active metabolites in human small intestinal and liver S9 fractions, cryopreserved enterocytes, cryopreserved hepatocytes in the presence or absence of human serum, or human serum were evaluated (Fig. 5). When benazeprilat and mycophenolic acid were incubated in hepatocytes in the absence of human serum for 240 min, they were decreased to 78.6% and 23.5%, respectively, whereas they were stable in hepatocytes in the presence of human serum. These results suggest that protein binding to serum might reduce the metabolism by decreasing the free concentration of compound. When 5-hydroxymethyl tolterodine, estramustine, amprenavir, and prednisolone were incubated in hepatocytes in the presence of human serum for 240 min, they were decreased to 76.3%, 30.4%, 58.7%, and 74.3%, respectively, whereas they were stable in hepatocytes in the absence of human serum. It was surmised that some components in human serum might enhance or catalyze the metabolic reactions. Estramustine and etoposide were unstable in human small intestine S9 fractions or enterocytes. Since estramustine phosphate, the prodrug of estramustine, was

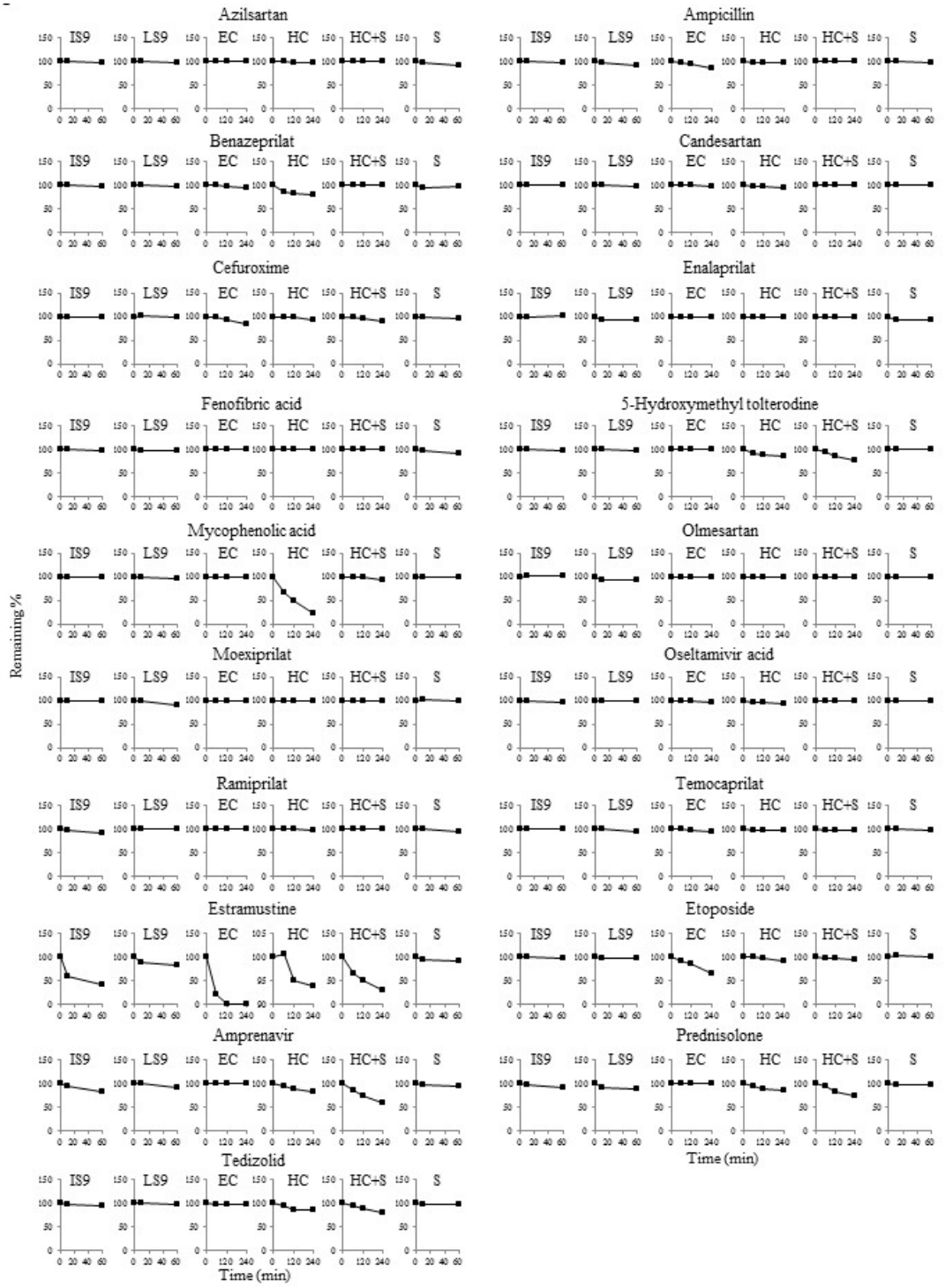


Fig. 5. Stabilities of 21 tested active metabolites in human various matrices. Test active metabolites (5 μM) were incubated with human small intestine S9 fractions (IS9), liver S9 fractions (LS9), enterocytes (EC), hepatocytes in the absence (HC) or presence of serum (HC + S), or serum (S). Open squares represent the percentages of active metabolites remaining.

highly stable in human small intestine S9 fractions and enterocytes (Fig. 4), estramustine appears to have been developed to avoid first-pass effects in the small intestine. The other active metabolites were relatively stable (over 80%) in all the human matrices. Because active metabolites should be relatively more stable to some extent than prodrugs, it might be effective to control the metabolism of the prodrugs when their conversion to active metabolites is too fast.

DISCUSSION

In drug development, the prodrug approach provides possibilities to overcome various obstacles such as low bioavailability, short duration of action, lack of site specificity, toxicity, and local irritation. The most common purpose of prodrug development is to enhance oral absorption, and many prodrugs are designed to improve poor permeability and/or solubility (Rautio et al., 2008; Huttunen ey al., 2011). Although the strategy is important to efficiently develop new prodrugs, the methodology for selecting candidate compounds based on the early-ADME data has not been established. Furthermore, it is difficult to predict drug disposition in humans from animal data, because the information regarding species differences in the specific activities, tissue distributions, and substrate specificities of hydrolases is still limited. The purpose of this study is to establish an *in vitro* system for selection of candidate prodrugs and appropriate experimental animals based on the physicochemical/biopharmaceutical properties and metabolic stability using 21 model prodrugs with improved membrane permeability/aqueous solubility.

Generally, it is well known that a compound with lower log D shows poor permeability, whereas that with higher log D shows poor solubility (Hendriksen et al., 2003). The differences in log D values of prodrugs with improved membrane permeability/aqueous solubility and those of their active metabolites were examined. The log D values of the prodrugs with improved membrane permeability were higher than those of their active metabolites (Fig. 1). On the other hand, the log D values of the prodrugs with improved

aqueous solubility were lower than those of their active metabolites. These results indicate that the membrane permeability and aqueous solubility of the 21 model prodrugs had actually been improved just as intended, and that log D value would be a good index for the design of prodrug.

To further characterize physicochemical properties of the tested compounds, their solubilities in JP1, JP2, FaSSIF, FeSSIF, or PBS were determined (Table 6). In the 16 prodrugs with improved membrane permeability, most prodrugs and their active metabolites showed solubilities of over 80%, but azilsartan medoxomil, candesartan cilexetil, and mycophenolate mofetil showed lower solubility than that of their active metabolite (Fig. 2). In the 5 prodrugs with improved aqueous solubility, estramustine phosphate and tedizolid phosphate showed higher solubility in buffers, except for JP1, than their active metabolites. Hence, artificial intestinal fluids can be used to evaluate the improvement of solubility of prodrugs with improved aqueous solubility. The lipophilicity of prodrugs with improved membrane permeability should be controlled so that the solubility of the prodrugs would not become extremely low, although the lipophilicity of prodrugs should be higher than that of their active metabolites.

Caco-2 cells are often used to predict the permeability of compounds in the human small intestine. This study evaluated the membrane permeability of 16 prodrugs with improved membrane permeability and their active metabolites using Caco-2 cells (Table 7). In 9 out of 16 prodrugs, the $P_{app\_PD\ total}$ values were higher than the P_{app_AM} values. Also, 10 out of 16 prodrugs showed higher $P_{app\_PD>AM}$ values than P_{app_PD} values. Among them, benazepril, ramipril, and temocapril are known to be hydrolyzed by CES1, which is expressed in Caco-2 cells but not in human enterocytes (Imai et al., 2005). The difference in CES1 expression would cause inconsistency between *in vitro* and *in vivo* evaluation. Membrane permeability was thus evaluated under the addition of BNPP, a general serine esterase inhibitor (Table 7). However, the $P_{app\_PD\ total}$ values of benazepril, moexipril, ramipril, and temocapril, which are hydrolyzed by CES1, were not changed by the addition of BNPP. That might be owing to involvement of an enzyme(s) that is not inhibited BNPP, or the possibility that the hydrolysis

might have proceeded during the incubation by partially remaining enzyme, because of highly efficient hydrolysis. The membrane permeability assay using Caco-2 cells, in which hydrolysis can be ignored, should be optimized for practical use. The membrane permeability assay using Caco-2 cells might not be suitable for the selection of compounds intended to proceed to *in vivo* PK study or development of a strategy for prodrugs. Application of other assays to evaluate membrane permeability, such as parallel artificial membrane permeability assay (PAMPA) and membrane permeability assay using Madin-Darby canine kidney (MDCK) cells, might be useful. However, since the membrane permeability is correlated with the log D values, the measurement of log D might be sufficient for the evaluation of the membrane permeability to decide whether to proceed with development.

Prior to the evaluation of the metabolic stability of the prodrugs, the extent of their non-enzymatic degradation, that is, the stability of prodrugs in buffers or media, was evaluated (Fig. 3). It was demonstrated that some prodrugs, including azilsartan medoxomil (Williams' E Medium), bacampicillin (all buffers and media), cefuroxime axetil (all buffers and media), fenofibrate (potassium phosphate buffer), lenampicillin (all buffers and media), olmesartan medoxomil (HBSS, and Williams' E Medium), and sultamicillin (all buffers and media) were non-enzymatically degraded. Therefore, some clinically used prodrugs appear to be non-enzymatically degraded in the body.

Next, the metabolic stability of the prodrugs and the formation rate of their active metabolites were evaluated by various human matrices (Fig. 4). Almost all the prodrugs were immediately hydrolyzed enzymatically and/or spontaneously. It was an unexpected finding that many of the prodrugs with improved membrane permeability were hydrolyzed within human intestinal epithelium cells. In general, prodrugs with improved membrane permeability should not be hydrolyzed in small intestine, to facilitate the increased bioavailability. However, even if a prodrug is hydrolyzed in small intestine, the formed active metabolites would be transported into enterocytes according to their physiological properties (Pang, 2003). Supporting the hypothesis, it has been reported that the hydrolysis of compounds in small intestine is not necessarily the reason for low bioavailability (Mizuma, 2010). Thus, the

hydrolysis in the small intestine would not be the sole factor to reject a candidate prodrug.

Metabolic stability was also evaluated using animal matrices. To examine the species differences in metabolic efficiency of the prodrugs, CR value, which represents the ratio of amount of the formed active metabolite to the decreased amount of prodrug (Table 8), and CS_{total} value, which represents the ratio of the apparent clearance for the active metabolite formation to the total clearance of prodrug (Table 9), were calculated. Although the tested prodrugs showed high CS_{total} values in humans, they showed lower CS_{total} values in monkey and dog. It is generally recognized that monkey shows similar tissue distribution and substrate specificities of drug-metabolizing enzymes, but *F* (especially F_g) of monkey is not always close to that of humans (Chiou and Buehler, 2002). Dog shows similar absorption to humans, but shows lower hydrolase activities than humans (Fukami and Yokoi, 2012; Chiou et al., 2000), with lacking CES2 (Yoshida et al., 2008). The CS_{total} values in rat were close to those in humans, although the numbers of CES isoforms and their expression levels in rat were different from those in humans (Bahar et al., 2012). Thus, the CS_{total} value might be a good parameter for selection of animals for *in vivo* PK screening to evaluate bioavailability showing similarity to humans.

A scheme for an *in vitro* screening of candidate prodrugs with sufficient oral absorption is depicted in Figure 6. In the 1st step, pharmacologically active compounds with low metabolic clearance are selected. In the 2nd step, the number of candidate prodrugs is narrowed down based on the criteria for log D, solubility in artificial intestinal fluids, membrane permeability, and human CS_{total}. In the 3rd step, animal species to be used in the subsequent *in vivo* PK screening is selected based on animal CS_{total}. In the 4th step, the *in vivo* PK in the selected experimental animal is evaluated. Based on the results from the *in vivo* PK analysis, in the 2nd step, and, if necessary, in the 1st step, would be reapplied to select other candidate prodrugs. This scheme would streamline the development of prodrugs.

In conclusion, the present study successfully proposed a novel screening strategy for efficient selection of prodrug candidates. This scheme would be a breakthrough in the prodrug development.

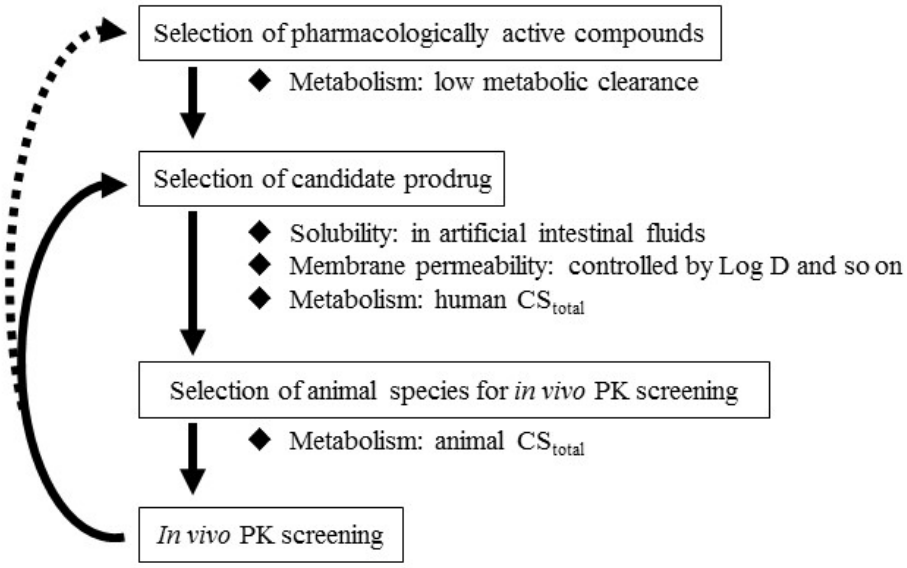


Fig. 6. Scheme for *in vitro* screening of candidate ptodrugs designed to enhance oral absorption.

CHAPTER 2

Systematic approach for selection of prodrugs: evaluation using oseltamivir analogues as models

SUMMARY

In Chapter 1, the systematic strategy for selecting prodrugs based on log D value, solubility in artificial intestine fluids, membrane permeability, and metabolic instability has been established. The purpose of this study is to evaluate the utility of this strategy using oseltamivir and 23 kinds of oseltamivir analogues having various types of side chain as well as their active metabolite, oseltamivir acid, as model compounds. Log D values of oseltamivir and analogues (2.0 to 4.9) were higher than that of oseltamivir acid (0.7), supporting a fact that oseltamivir had been developed to improve permeability of oseltamivir acid. Solubilities of analogues in artificial intestinal fluids were over 80% except a compound with the highest lipophilicity. A positive correlation was observed between membrane permeability and log D values of analogues. In metabolic profiles, species differences in the hydrolysis efficiency were observed depending on analogues. By using the systematic strategy, it was demonstrated that oseltamivir and some analogues are appropriate prodrugs which could be proceeded to *in vivo* pharmacokinetic studies, with selection of suitable animals. This study could confirm the utility of the systematic strategy for the prodrug development.

INTRODUCTION

In Chapter 1, a systematic strategy has been established for selecting candidate prodrugs by using 21 model prodrugs, focusing on the following 4 items: 1) log D, 2) solubility in artificial intestinal fluids, 3) membrane permeability, and 4) metabolic instability.

Log D values can be used as an index of lipophilicity so that prodrugs with improved membrane permeability should be more lipophilic than its active metabolites. Solubility of prodrugs with improved membrane permeability in artificial intestinal fluids would not be extremely low compared with its active metabolites. Membrane permeability of prodrugs should be higher than that of its active metabolites. After the absorption, prodrugs should be efficiently converted to an active metabolite, and metabolic profile in small intestine should be ideally similar between human and experimental animals. The used 21 model compounds were from clinically used prodrugs (with different structures); therefore, they should meet the criteria for desirable prodrugs. To evaluate the utility of the systemic approach for selecting candidate prodrugs, the use of model compounds with a basic structure (active metabolite) and varied side chains, having varied physicochemical characteristics, might be better.

Oseltamivir, an anti-influenza drug, is a prodrug of oseltamivir acid (Zaraket and Saito, 2016). Oseltamivir had been developed to improve permeability of oseltamivir acid, of which bioavailability is less than 5%, resulting in over 80% bioavailability in human (He et al., 1999). After the absorption, oseltamivir is efficiently hydrolyzed to oseltamivir acid. In Chapter 2, to evaluate the utility of the systemic approach for selecting candidate prodrugs had been previously developed, physicochemical/biopharmaceutical properties and metabolic profiles of oseltamivir analogues with various substituted side chains in the ester group of oseltamivir were determined. For the construction of analogues, they were designed so that these compounds have higher log D values than oseltamivir acid with varied lipophilicities, and preference to be hydrolyzed by CES 2, which is expressed in the human intestine and liver, although oseltamivir is hydrolyzed by CES 1 expressed in the liver (Oda at al., 2015). By characterizing of physicochemical and metabolic properties of 23 kinds of constructed oseltamivir analogues and oseltamivir, the utility of the systemic approach for selecting candidate prodrugs was evaluated.

EXPERIMENTAL PROCEDURES

Chemicals and reagents

Oseltamivir was purchased from FUJIFILM Wako Pure Chemical (Osaka, Japan). Oseltamivir acid was purchased from Toronto Research Chemicals (Toronto, Canada). All other chemicals used in this study were of analytical or the highest quality commercially available. Cryopreserved enterocytes, cryopreserved hepatocytes, and sera from four species (human, rat, monkey, and dog) shown in Table 10 were purchased from In Vitro ADMET Laboratories (Columbia, MD), BioIVT (Westbury, NY), and Biopredic International (Rennes, France), respectively.

Synthesis of 23 kinds of oseltamivir analogues

Twenty-three oseltamivir analogues shown in Fig. 7 were synthesized in Japan Tobacco Inc (Osaka, Japan). These analogues were designed to have a varied side chain on an alcohol group of oseltamivir acid to investigate compounds with various lipophilicities. In addition, these analogues were designed to have higher predicted log D values than oseltamivir acid, because oseltamivir was originally developed to improve membrane permeability of oseltamivir acid. Some analogues were designed to be hydrolyzed by CES2, which is expressed in the small intestine, to investigate the effect of small intestinal metabolism on membrane permeability.

Determination of log D, solubility, and membrane permeability using Caco-2 cells

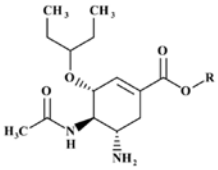
The log D, solubility, and membrane permeability of test compounds using Caco-2 cells were evaluated according to the method in Chapter 1. The used LC/MS and LC-MS/MS apparatus were also similar to Chapter 1. MS conditions for individual analytes are shown in Table 11.

Table 10. Donor information of various matrices.

Species	Strain, breed or race	Sex (animals or individuals)	Age	Lot No.
Human				
Cryopreserved enterocytes	C	1 F	44 years	HE3009
Cryopreserved hepatocytes	C (17), H (2), and O (1)	Pool of 10 M and 10 F	3 Days – 80 years	UYG
Serum	Not available	Pool of 13 M and 13 F	27 - 68 years	SER019A050C001
Rat				
Cryopreserved enterocytes	Sprague-Dawley	Pool of 12 M	7 - 10 weeks	RSE3092
Cryopreserved hepatocytes	Sprague-Dawley	Pool of 20 M	7 - 8 weeks	SNQ
Serum	Sprague-Dawley	Pool of 70 M and 35 F	10 – 19 weeks	5H1552
Dog				
Cryopreserved enterocytes	Beagle	1 M	3 – 4 months	DBE3026
Cryopreserved hepatocytes	Beagle	Pool of 3 M	7 months	GRQ
Serum	Beagle	Pool of 10 M and 5 F	3 – 6 years	6G1423
Cynomolgus monkey				
Cryopreserved enterocytes	--	Poor of 3 M	3 - 4 years	PCE3091
Cryopreserved hepatocytes	--	Pool of 3 M	4 - 6 years	HXW
Serum	--	Pool of 21 M and 13 F	20 months – 74 months	5K2003

C: Caucasian, H: Hispanic, O: Oriental, M: male, F: female, (): Number of donors.

--: Not applicable



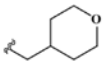
Compound	R	Compound	R
Oseltamivir acid	H	Compound K	$CH_2CH_2CH_3$
Oseltamivir	CH_2CH_3	Compound L	C_6H_6
Compound A	CH_3	Compound M	$C(CH_3)_3$
Compound B	$CH_2CH_2OCH_3$	Compound N	$CH_2CH(CH_3)_2$
Compound C	CH_2OCOCH_3	Compound O	$CH_2C_6H_6$
Compound D		Compound P	$CH_2CH_2CH_2CH_3$
		Compound Q	$CH_2C(CH_3)_3$
Compound E		Compound R	$CH_2CH_2CH(CH_3)_2$
		Compound S	C_6H_{12}
Compound F	$CH(CH_3)CH_2OCH_3$	Compound T	
Compound G	$CH(CH_3)OCOCH_3$		
Compound H	$CH_2CH_2CH_2CH_2OCH_3$	Compound U	$CH_2CH_2CH_2CH_2CH_2CH_3$
Compound I	$CH_2CH_2CH(CH_3)OCH_3$	Compound V	$CH_2C_6H_{12}$
Compound J	$CH(CH_3)_2$	Compound W	$CH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_3$

Fig. 7. Chemical structures of oseltamivir and its analogues used in this study.

Parallel artificial membrane permeability assay (PAMPA)

PAMPA was performed by using Corning Gentest Pre-Coated PAMPA Plate System (Corning, Bedford, MA) with either transport buffer pH 5.5 or pH 7.4, which is HBSS containing 20 mM 2-(*N*-morpholino) ethanesulfonic acid or 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid. Oseltamivir, 23 kinds of oseltamivir analogues, and oseltamivir acid were each dissolved in DMSO as a stock solution (10 mM). The compound solutions diluted with the transport buffer by 200 fold (final concentrations: 50 μM) were added to the well (300 μL/well) of the receiver plate, and the transport buffer was added to the well (200 μL/well) of the filter plate. The filter plate was then coupled with the receiver plate. After 5-h incubation at room temperature, the concentrations of oseltamivir and its analogues as well as oseltamivir acid formed by spontaneous decomposition in both plates were measured. Equilibrium concentration ($C_{equilibrium}$), permeability coefficients (P_e), and membrane retention (R), which represents the binding to the plate and/or membrane, were

Table 11. LC-MS/MS conditions for compounds with detailes on mass transitions.

Compound	m/z	Collision Energy
Oseltamivir acid	285.11 > 197.08	10
Oseltamivir	313.65 > 166.07	20
Compound A	299.00 > 151.91	20
Compound B	357.05 > 119.90	30
Compound C	371.04 > 136.99	20
Compound D	383.10 > 119.91	30
Compound E	361.04 > 272.98	10
Compound F	371.08 > 119.91	30
Compound G	367.10 > 137.89	30
Compound H	357.02 > 119.90	30
Compound I	371.08 > 119.92	30
Compound J	341.06 > 137.91	20
Compound K	341.07 > 193.99	20
Compound L	357.07 > 90.77	30
Compound M	327.05 > 179.97	20
Compound N	355.13 > 208.04	20
Compound O	379.08 > 137.90	30
Compound P	355.08 > 208.03	20
Compound Q	341.07 > 194.00	20
Compound R	369.08 > 222.03	20
Compound S	381.12 > 234.04	20
Compound T	357.05 > 119.88	30
Compound U	397.12 > 250.08	20
Compound V	369.07 > 281.01	10
Compound W	343.05 > 119.88	30

calculated as follows:

$$C_{equilibrium}\ (\text{mM}) = [C_D(t) \times V_D + C_A(t) \times V_A] / (V_D + V_A)$$

$$P_e\ (\text{cm/s}) = \{-\ln[1-C_A(t) / C_{equilibrium}]\} / [A \times (1/V_D + 1/V_A) \times t]$$

$$R = 1- [C_D(t) \times V_D + C_A(t) \times V_A] / (C_0 \times V_D)$$

where C_0 and $C_D(t)$ are the concentration of compound in donor well at time 0 and t, respectively, $C_A(t)$ is the concentration of compound in acceptor well at time t, V_D and V_A are the volumes of donor and acceptor well (0.3 and 0.2 mL), respectively, t is the incubation time (18,000 s), A is the membrane surface area (0.3 cm^2/well), and R is mass retention.

Evaluation of metabolic stability of oseltamivir analogues

The metabolic stabilities of oseltamivir and their analogues were evaluated using cryopreserved enterocytes, cryopreserved hepatocytes, sera, and bactosomes expressing human CES1 or CES2 according to the method in Chapter 1. A typical incubation mixture was the same as Chapter 1 except for the cell density of enterocytes at 0.5×10^6 cells/mL and hepatocytes at 0.1 to 1.0×10^6 cells/mL, and protein concentration of bactosomes at 0.5 mg/mL (Table 12). The mixtures containing enterocytes and hepatocytes were incubated for 0 to 240 min at 37°C in a CO_2 incubator. The mixtures containing serum were incubated for 0, 5, 30, and 60 min at 37°C. The mixtures containing bactosomes were incubated for 0, and 30 min at 37°C. The conditions for mass spectrometry of individual analytes are shown in Table 13.

Table 12. Conditions of metabolism studies.

Enzyme source	Incubation time (min)	Compound
Enterocytes from human, rat, monkey, and dog		
0.5×10^6 cells/mL	0, 10, 60, 240	All compounds
Hepatocytes with 10% serum from human		
0.1×10^6 cells/mL	0, 1, 3, 10	Compound C, G, L, and U
0.2×10^6 cells/mL	0, 10, 60, 120	Oseltamivir, Compound A, B, D, F, J, K, N, O, P, R, S, T, V, and W
0.5×10^6 cells/mL	0, 60, 120, 240	Compound H, I, M, and Q
1.0×10^6 cells/mL	0, 60, 120, 240	Compound E
Hepatocytes with 10% serum from rat		
0.1×10^6 cells/mL	0, 0.5, 1, 3	Oseltamivir, Compound A, B, D, E, F, H, I, J, M, Q, S, and T
0.2×10^6 cells/mL	0, 10, 60, 120	Compound C, G, K, L, N, O, P, R, U, V and W
Hepatocytes with 10% serum from monkey		
0.1×10^6 cells/mL	0, 3, 10, 60	Compound C
0.2×10^6 cells/mL	0, 10, 60, 120	Oseltamivir, Compound B, F, G, I, J, K, L, N, O, P, Q, R, S, T, U, V, and W
0.5×10^6 cells/mL	0, 60, 120, 240	Compound A, D, E, H, and M
Hepatocytes with 10% serum from dog		
0.1×10^6 cells/mL	0, 3, 10, 60	Compound C
0.2×10^6 cells/mL	0, 10, 60, 120	Compound G, L, N, R, S, T, U, V, and W
0.5×10^6 cells/mL	0, 60, 120, 240	Oseltamivir, Compound A, B, D, E, F, H, I, J, K, M, O, P, and Q
Serum from human, rat, monkey, and dog		
10%	0, 5, 30, 60	All compounds
Bactosomes		
0.5 mg/mL	0, 30	All compounds

Table 13. LC-MS/MS conditions for compounds.

Compound	m/z	Collision energy
Oseltamivir acid	285.11 > 197.08	10
Oseltamivir	313.65 > 166.07	20
Compound A	299.00 > 151.91	20
Compound B	357.05 > 119.90	30
Compound C	371.04 > 136.99	20
Compound D	383.10 > 119.91	30
Compound E	361.04 > 272.98	10
Compound F	371.08 > 119.91	30
Compound G	367.10 > 137.89	30
Compound H	357.02 > 119.90	30
Compound I	371.08 > 119.92	30
Compound J	341.06 > 137.91	20
Compound K	341.07 > 193.99	20
Compound L	357.07 > 90.77	30
Compound M	327.05 > 179.97	20
Compound N	355.13 > 208.04	20
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Compound P	355.08 > 208.03	20
Compound Q	341.07 > 194.00	20
Compound R	369.08 > 222.03	20
Compound S	381.12 > 234.04	20
Compound T	357.05 > 119.88	30
Compound U	397.12 > 250.08	20
Compound V	369.07 > 281.01	10
Compound W	343.05 > 119.88	30

RESULTS

Log D values and solubility of oseltamivir acid, oseltamivir, and its analogues in various buffers

To determine the physicochemical properties of oseltamivir and its analogues as well as oseltamivir acid, their log D values and solubilities in JP1, JP2, FaSSIF, FeSSIF, and PBS were evaluated (Table 14). The log D values of oseltamivir and its analogues (2.0 to 4.9) were higher than that of oseltamivir acid (0.7), suggesting that oseltamivir and its analogues have preferable lipophilicity. The solubility of most test compounds was over 80% in all buffers,

except compound W (73.6% and 53.1% in JP2 and FaSSIF, respectively), which shows the highest lipophilicity (Log D = 4.9). Therefore, the compound W unlikely be selected as a prodrug with high priority.

Table 14. The log D values and solubilities (%) of oseltamivir acid, oseltamivir, and its analogues.

Test compounds	Log D	JP1	JP2	FaSSIF (%)	FeSSIF	PBS
Oseltamivir acid	0.7	100	99	96	98	100
Oseltamivir	2.4	97	100	89	95	100
Compound A	2.0	100	100	100	100	100
Compound B	2.1	100	100	100	81	100
Compound C	2.2	100	100	100	89	100
Compound D	2.2	100	96	100	91	100
Compound E	2.4	100	100	100	100	100
Compound F	2.5	100	100	100	99	100
Compound G	2.6	93	100	100	100	100
Compound H	2.6	100	100	100	100	100
Compound I	2.6	100	100	100	100	100
Compound J	2.8	100	100	100	100	99
Compound K	2.8	98	100	95	96	93
Compound L	3.1	99	100	100	100	100
Compound M	3.1	100	100	100	100	100
Compound N	3.2	97	100	94	100	100
Compound O	3.2	100	99	98	97	98
Compound P	3.2	100	100	96	100	100
Compound Q	3.5	100	100	100	100	96
Compound R	3.6	100	96	92	96	91
Compound S	3.6	100	100	98	100	96
Compound T	3.7	100	100	100	100	100
Compound U	4.1	100	93	98	100	92
Compound V	4.1	100	100	100	100	100
Compound W	4.9	100	74	53	100	82

Membrane permeability assays of oseltamivir and its analogues

To evaluate the membrane permeability of oseltamivir and its analogues, their P_{app_total} values were calculated by membrane permeability assay using Caco-2 cells, and P_e values of them were calculated by PAMPA. The P_{app_total} value represents the sum of P_{app} values of oseltamivir or its analogues and P_{app} value of the formed oseltamivir acid. As shown in Fig. 8A, the P_{app_total} value of oseltamivir and its analogues were 0.03×10^{-6} to 0.89×10^{-6} cm/s, and these values were correlated with their log D values ($r = 0.78$, $p < 0.05$). The P_{app_total} value of oseltamivir (0.10×10^{-6} cm/s) was close to that of oseltamivir acid (0.11×10^{-6} cm/s), even though oseltamivir had been developed to improve membrane permeability of oseltamivir acid. Although the log D values of all analogues were higher than that of oseltamivir acid, the P_{app_total} value of 8 out of 23 analogues were lower than that of oseltamivir acid. In PAMPA, the P_e values of the compounds under the pH 5.5 (Fig. 8B) or pH 7.4 (Fig. 8C) were evaluated according to a previous report (Bermejo et al., 2004), considering the effects of ionization on the membrane permeability. The pKa value of oseltamivir and its analogues was all 9.3. Since the R values of all test compounds were below 0.5 (data not shown), it was considered that the binding of these compounds to the plate and/or membrane was not so high to affect the evaluation of the P_e values. Under the pH 5.5 condition, the P_e values of oseltamivir and its analogues were 0.18×10^{-5} cm/s and 0.09 to 4.4 $\times 10^{-5}$ cm/s, respectively, whereas the value of oseltamivir acid was not obtained because it was not detected in acceptor well. As shown in Fig. 8B, the P_e values were correlated with the log D values ($r = 0.72$, $p < 0.05$). Under the pH 7.4 condition, the P_e values of oseltamivir acid, compound C, and compound W were not obtained because they were not detected in acceptor wells. The P_e values of oseltamivir and the other analogues were 0.59×10^{-5} cm/s and 0.17×10^{-5} to 19.2×10^{-5} cm/s, respectively, showing a significant correlation with the log D values ($r = 0.60$, $p < 0.05$) (Fig. 2C). The P_e values of the analogues except for compounds B, C, and W under the pH 7.4 condition were higher than that of them under the pH 5.5 condition. Unlike the membrane permeability assay using Caco-2 cells, PAMPA demonstrated that oseltamivir has higher membrane permeability than oseltamivir acid. For these cases

presented here, PAMPA is likely more suitable for evaluation of membrane permeability than the assay using Caco-2 cells. In addition, since it has been known that the P_e value is well-correlated with lipophilicity, log D value might be better index for membrane permeability.

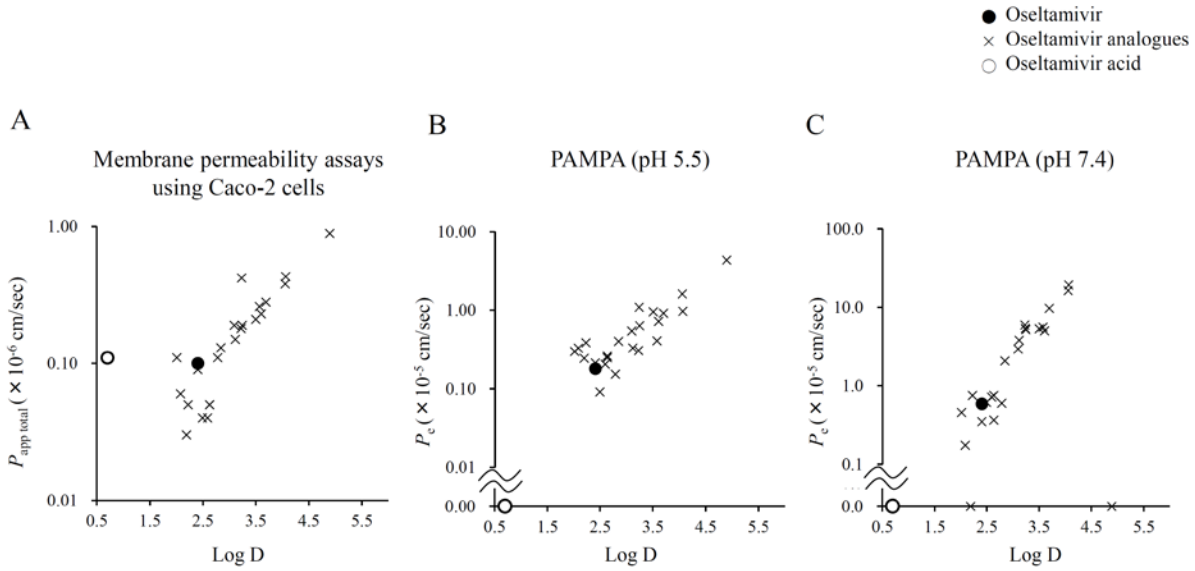

Fig. 8. Relationship between log D and (A) $P_{app\ total}$ in membrane permeability assay using Caco-2 cells, (B) P_e in PAMPA (pH 5.5), or (C) P_e in PAMPA (pH 7.4) of oseltamivir, its analogues, and oseltamivir acid.

Stabilities of oseltamivir and its analogues in buffers or media

To investigate the extent of spontaneous degradation of oseltamivir and its analogues, their stabilities in Williams' E Medium (used for metabolism by cryopreserved enterocytes), HBSS (used for metabolism by cryopreserved hepatocytes), and potassium phosphate buffer (used for metabolism by serum) were evaluated (Fig. 9). Oseltamivir and its analogues at 5 μM were incubated for 8 h. Compound C and compound G were unstable, as their remaining percentages were 0% and 12% in Williams' E Medium, 38% and 74% in HBSS, and 45% and 77% in potassium phosphate buffer, respectively. Compound L showed relatively low instability in Williams' E Medium and HBSS with 42% and 74% remaining percentages, respectively. Thus, oseltamivir analogues having (acyloxy)alkyl or phenyl ester appear to be non-enzymatically degraded. The other test compounds were stable in any

buffers, showing over 80% remaining percentages.

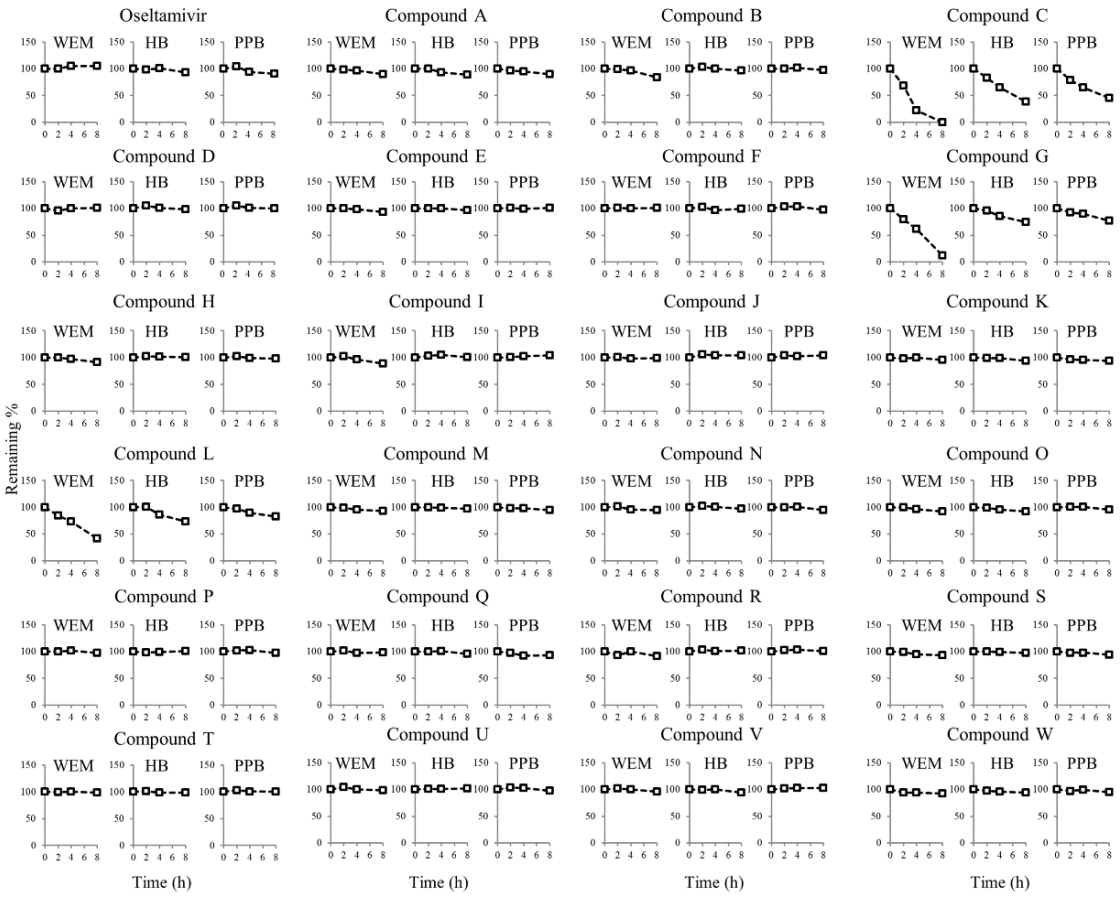

Fig. 9. Test compounds (5 μM) were incubated in Williams' E Medium (WEM), HBSS (HB), or potassium phosphate buffer (PPB) for 8 h.

Metabolic stabilities of oseltamivir and its analogues by various human and animal matrices

The metabolic stabilities of oseltamivir or its analogues as well as the enzymatic formation of oseltamivir acid from them were examined using human enterocytes, hepatocytes in the presence of human serum, or human serum (Fig. 10). The remaining percentages of oseltamivir in enterocytes (at 240 min), hepatocytes (at 120 min), and serum (at 60 min) were 100%, 65%, and 96%, respectively. The amount of the formed oseltamivir acid was equivalent to the amount of the decreased oseltamivir, keeping mass balance. The remaining percentages of compounds C, G, and L incubated with enterocytes (0% to 74%),

hepatocytes (48% to 89%) or serum (0% to 89%) were lower than those in corresponding buffers (Williams' E Medium: 68.0% to 84.2%, HBSS: 83% to 101%, and potassium phosphate buffer: 79% to 97%), suggesting that these compounds are hydrolyzed by all matrices. The remaining percentages of compound D by all matrices were over 90%. As for the other compounds, the remaining percentages by hepatocytes were below 90%, whereas those by serum and enterocytes were over 90%, indicating that they are hydrolyzed in hepatocytes. This tendency was similar to oseltamivir. It is known that oseltamivir is hydrolyzed by CES1, which is abundantly expressed in the human liver but hardly expressed in the small intestine or blood (Bahar et al., 2012). Therefore, it was surmised that oseltamivir

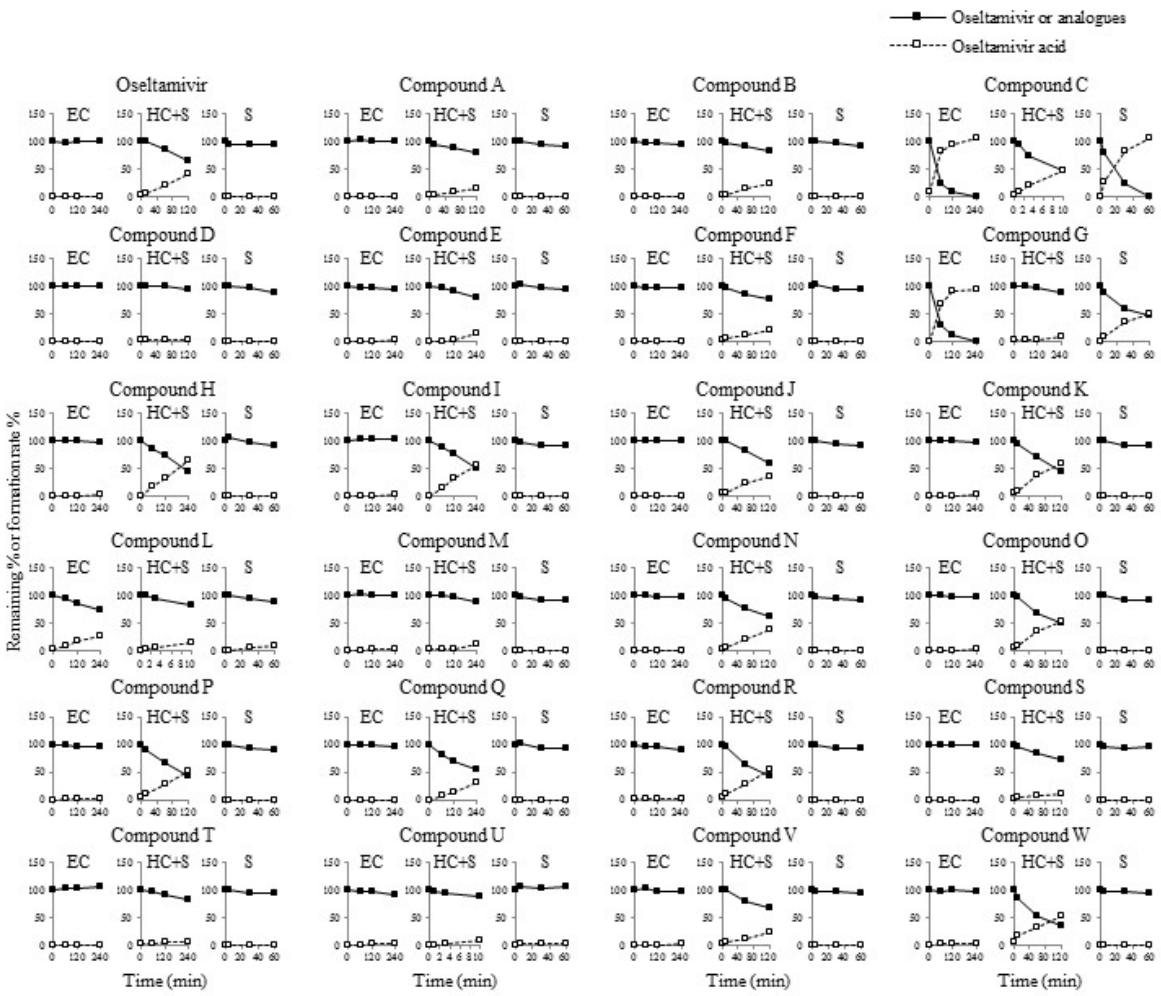

Fig. 10. Stabilities of oseltamivir and its analogues in human matrices. Test compounds (5 μM) were incubated with human enterocytes (EC), hepatocytes in the presence of serum (HC + S), or serum (S). Closed squares represent the remaining percentages of oseltamivir or its analogues and open squares represent the percentages of the formed oseltamivir acid.

analogues except for compound C, G, and L might be hydrolyzed by CES1. The $CR_{small\ intestine}$, CR_{liver}, and CR_{serum} values were calculated from the metabolic stability assays using enterocytes, hepatocyte, and serum, respectively (Table 15). The CR value of compound D could not be calculated because of high metabolic stability. The CR values of compounds C, G, and L in all tissues were 0.9 to 1.0, indicating that major metabolite of them was oseltamivir acid. Since the CR_{liver} values of oseltamivir, compounds A, B, F to P, and R were 0.9 to 1.0, it was revealed that oseltamivir acid was major metabolite of them. On the other hand, the CR_{liver} values of compounds E, Q, and S to W were 0.3 to 0.8, indicating that they are converted to not only oseltamivir acid but also other metabolite(s) in the liver. The CS_{total} values of the analogues were similar to CR values because most of the analogues were hydrolyzed in only liver. The CS_{total} values of compound S (0.4) and T (0.3) were extremely low, and they unlikely be selected as a prodrug with high priority.

To investigate the species differences in metabolic efficiency of the analogues, the same assays were performed using enterocytes, hepatocytes with serum, and serum from rat, dog, and monkey (Table 15). In rat, the $CR_{small\ intestine}$ values of compounds C, G, and L were 1.0, as with the case of human. $CR_{small\ intestine}$ values of compounds N to P, R, S, and U to W were 0.9 to 1.0, indicating that they are efficiently converted to oseltamivir acid in rat, showing great difference from human. The CR_{liver} value of compound D was 1.0, although that in human could not be calculated because of high metabolic stability. Moreover, the CR_{liver} values of compound S and T (0.9 to 1.0) were higher than that in human (0.3 to 0.4). On the other hand, the CR_{liver} value of compound M (0.3) was lower than that in human (1.0). The hydrolysis in the serum were observed for all analogues with high CR_{serum} values (0.7 to 1.0), although only three compounds were hydrolyzed in human serum. Since rat serum contains Ces1c, although human, dog, and monkey plasma do not contain any CES isoforms (Oda et al., 2015), Ces1c might be responsible for their hydrolysis in rat serum.

In dog, the CR value of compound D could not be calculated because of high metabolic stabilities, as the case of human. The $CR_{small\ intestine}$ values (0.9 to 1.0) of compound C, G, and L were similar to those in human. Interestingly, compound W was metabolized in the dog

Table 15. Conversion ratio (CR) and conversion score (CS_{total}) of oseltamivir and its analogues in human and animal matrices.

Test compounds	Human				Rat				Dog				Monkey			
	CR			CS_{total}	CR			CS_{total}	CR			CS_{total}	CR			CS_{total}
	Small intestine	Liver	Serum		Small intestine	Liver	Serum		Small intestine	Liver	Serum		Small intestine	Liver	Serum	
Oseltamivir	--	1.0	--	**1.0**	--	1.0	1.0	**1.0**	--	1.0	--	**1.0**	--	0.8	--	**0.8**
Compound A	--	0.9	--	**0.9**	--	1.0	0.8	**0.8**	--	1.0	--	**1.0**	--	0.9	--	**0.9**
Compound B	--	1.0	--	**1.0**	--	1.0	1.0	**1.0**	--	1.0	--	**1.0**	--	1.0	--	**1.0**
Compound C	1.0	0.9	1.0	**1.0**	1.0	1.0	1.0	**1.0**	1.0	1.0	1.0	**1.0**	1.0	1.0	0.9	**0.9**
Compound D	--	--	--	--	--	1.0	1.0	**1.0**	--	--	--	--	--	1.0	--	**1.0**
Compound E	--	0.7	--	**0.7**	--	1.0	1.0	**1.0**	--	0.1	--	0.1	--	0.4	--	0.4
Compound F	--	1.0	--	**1.0**	--	1.0	1.0	**1.0**	--	0.8	--	**0.8**	--	0.8	--	**0.8**
Compound G	0.9	1.0	0.9	**0.9**	1.0	0.9	1.0	**0.9**	1.0	1.0	0.9	**0.9**	1.0	0.9	0.8	**0.8**
Compound H	--	1.0	--	**1.0**	--	1.0	1.0	**1.0**	--	1.0	--	**1.0**	--	1.0	--	**1.0**
Compound I	--	1.0	--	**1.0**	--	1.0	1.0	**1.0**	--	1.0	--	**1.0**	--	0.8	--	**0.8**
Compound J	--	0.9	--	**0.9**	--	1.0	0.9	**0.9**	--	1.0	--	**1.0**	--	0.9	--	**0.9**
Compound K	--	1.0	--	**1.0**	--	1.0	1.0	**1.0**	--	1.0	--	**1.0**	--	0.9	--	**0.9**
Compound L	1.0	0.9	0.9	**0.9**	1.0	1.0	1.0	**1.0**	0.9	1.0	--	**1.0**	1.0	0.9	--	**0.9**
Compound M	--	1.0	--	**1.0**	--	0.3	0.7	0.6	--	0.9	--	**0.9**	--	0.3	--	0.3
Compound N	--	1.0	--	**1.0**	0.9	1.0	1.0	**1.0**	--	0.8	--	**0.8**	--	1.0	--	**1.0**
Compound O	--	1.0	--	**1.0**	1.0	0.9	1.0	**1.0**	--	1.0	--	**1.0**	1.0	1.0	--	**1.0**
Compound P	--	0.9	--	**0.9**	1.0	1.0	1.0	**1.0**	--	0.9	--	**0.9**	--	0.9	--	**0.9**
Compound Q	--	0.7	--	**0.7**	--	1.0	0.9	**0.9**	--	0.2	--	0.2	--	0.4	--	0.4
Compound R	--	1.0	--	**1.0**	1.0	1.0	1.0	**1.0**	--	0.8	--	**0.8**	--	0.7	--	**0.7**
Compound S	--	0.4	--	0.4	1.0	0.9	0.9	**0.9**	--	0.2	--	0.2	--	0.3	--	0.3
Compound T	--	0.3	--	0.3	--	1.0	1.0	**1.0**	--	0.0	--	0.0	--	0.4	--	0.4
Compound U	--	0.7	--	**0.7**	1.0	1.0	1.0	**1.0**	--	0.7	--	**0.7**	1.0	0.6	--	**0.7**
Compound V	--	0.7	--	**0.7**	0.9	1.0	1.0	**1.0**	--	0.2	--	0.2	--	0.6	--	0.6
Compound W	--	0.8	--	**0.8**	1.0	1.0	1.0	**1.0**	0.0	0.4	--	0.3	1.0	0.6	--	**0.7**

--: Due to high metabolic stability (remaining % of prodrugs were >90%), CR and CS_{total} could not be calculated.

small intestine to metabolite other than oseltamivir acid. The CR_{liver} values (0.1 to 0.4) of compound E, Q, V, and W were lower than that in human (0.7 to 0.8). The CR_{serum} values (0.9 to 1.0) of compound C and G were similar to human, whereas compound L was not metabolyzed in dog serum in contrast to the case of human.

In monkey, the $CR_{small\ intestine}$ values of compounds C, G, and L were 1.0, as with the case of human. In addition, the $CR_{small\ intestine}$ values of compounds O, U, and W were 1.0. The CR_{liver} value of compound D was 1.0. The CR_{liver} values of compounds E, M, Q, and V (0.3 to 0.6) were lower than those in human (0.7 to 1.0). The CR_{serum} values of compounds C and G (0.8 to 0.9) were close to those in human, whereas compound L was not metabolized in monkey serum as with the case of dog. Taken together, oseltamivir analogues used in this study are efficiently hydrolyzed in rat. In dog and monkey, some compounds were subjected to metabolic pathway(s) other than hydrolysis. Thus, *in vitro* metabolism study would be necessary for the selection of experimental animal suitable for the prediction of pharmacokinetics in humans.

Hydrolase activities for oseltamivir and its analogues by bactosomes expressing human CES1 and CES2

To clarify whether oseltamivir and its analogues are hydrolyzed by CES1 or CES2, metabolism study using recombinant enzymes was performed (Fig. 11). Oseltamivir was hydrolyzed by only recombinant human CES1, supporting the previous report (Oda et al., 2015). Compounds A, B, D, F, I to L, and N to W were also hydrolyzed by only CES1; therefore, that was consistent with the finding that their hydrolysis were observed in only hepatocytes, because CES1 is expressed in the liver, not in intestine and serum (Oda et al., 2015). Interestingly, compound C and compound G (with (acyloxy)alkyl) were efficiently hydrolyzed by CES2, in addition to CES1; therefore, the efficient hydrolysis of these compounds in enterocytes (Fig. 10) would be due to the hydrolysis by CES2, because CES2, but not CES1, is expressed in the intestine (Oda et al., 2015). Since CES2 did not catalyze the hydrolysis of compound L, which was moderately hydrolyzed in enterocytes, the hydrolysis

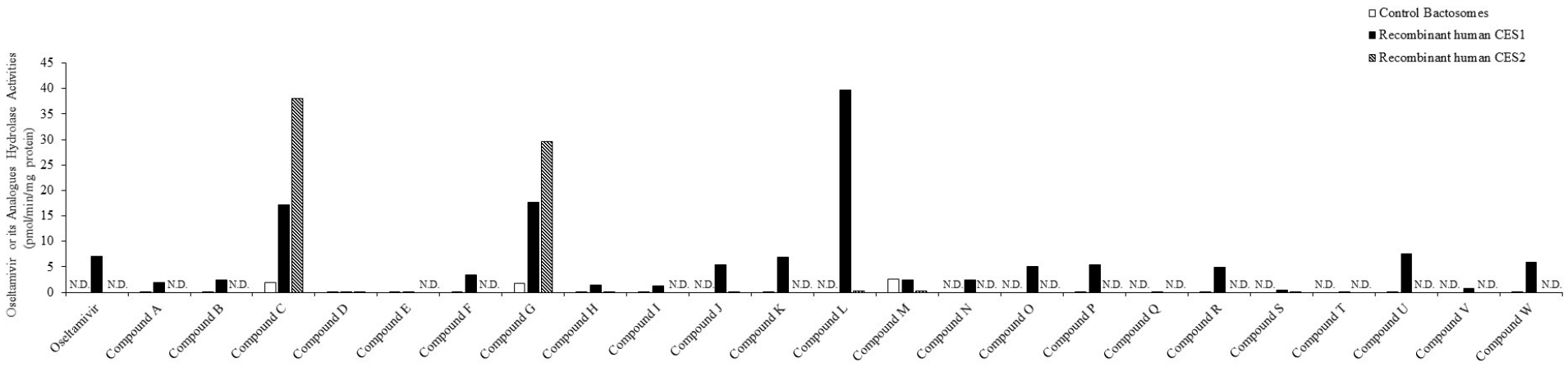

Fig. 11. The hydrolase activities for oseltamivir and its analogues (5 μM) by bactosomes expressing human CES1 and CES2.

of compound L in enterocytes would be owing to other enzyme(s). The hydrolysis of compounds C, G, and L in human serum would be catalyzed by enzymes other than CES enzymes, which are absent in serum (Bahar et al., 2012). Compounds E and M were not hydrolyzed by CES1 and CES2; therefore, their hydrolysis in hepatocytes would be owing to other enzyme(s). By comparison of hydrolase activities for oseltamivir, compound J and compound M by CES1, it was suggested that steric hindrance appears to be a factor of the decrease in the hydrolase activity. Similar trend was observed in compounds K, N, and Q. The result that the hydrolase activity of compound S was lower than that of compound L would be due to the structural difference that cyclohexane, but not benzene, is sterically distorted. In addition, the result that the hydrolase activity of compound O was lower than that of compound L would be due to the structural feature that the insertion of a carbon between the benzene ring and the ester group allows rotation of the benzene ring. Although it was suggested that some analogues are hydrolyzed by enzymes other than CES1 as intended, most of the analogues except for compounds C and G are mainly hydrolyzed by CES1 despite of varied functional groups. A function group with larger steric hindrance might be unsuitable for addition to the oseltamivir acid because it might decrease hydrolase activity and give a preference of metabolic reaction(s) other than hydrolysis.

DISCUSSION

Development of prodrug is one of the useful strategies for overcoming various defects of compounds such as low bioavailability, short duration of action, and toxicity (Akhani et al., 2017; Huttunen et al., 2011). In fact, approximately 10% of marketed drugs are prodrugs (Rautio et al., 2018). In Chapter 1, a novel systematic approach for screening of effective prodrugs based on physicochemical and ADME properties, which would be useful in early stage of drug development, has been established. Since this approach has been established using commercially available prodrugs as model compounds, which should meet the desired prodrug criteria, it was uncertain whether “undesired prodrug” can be omitted by using this

approach. The purpose of this study is to evaluate the utility of the established approach using oseltamivir analogues having varied function groups in the ester group of oseltamivir, considering the differences in substrate preference of CES1 and CES2 in the designing of analogues.

To evaluate the utility of the systematic approach for screening of effective prodrugs, 23 kinds of oseltamivir analogues shown in Fig. 7 were synthesized with varied lipophilicities (log D values). The log D values were from 2.0 in compound A to 4.9 in compound W. To characterize the physicochemical properties of the analogues, their solubilities in JP1, JP2, FaSSIF, FeSSIF, or PBS were evaluated. The analogues except for compound W showed a solubility with over 80% in all buffers (Table 14). Compound W in JP2 (76%) and FaSSIF (53%) showed a solubility below 80%, owing to high lipophilicity. Therefore, compound W might have a lower priority for development than the other analogues from the viewpoint of solubility.

Membrane permeabilities of oseltamivir and its analogues as well as oseltamivir acid were evaluated by using Caco-2 cells and PAMPA (Fig. 8). The P_{app_total} (assay using Caco-2 cells) or P_e (PAMPA) values of oseltamivir and its analogues were significantly correlated with and the log D values of them. Since the P_{app_total} values of oseltamivir and oseltamivir acid were almost equal, whereas P_e value of oseltamivir was higher than that of oseltamivir acid, it was suggested that PAMPA would be appropriate to evaluate the membrane permeability at least for oseltamivir analogues. Moreover, since the P_e values of all of the synthesized oseltamivir analogues were higher than that of oseltamivir acid, it was demonstrated that all analogues had better membrane permeability than oseltamivir acid.

Prior to the evaluation of the metabolic stability of the analogues, their stability in buffers or medium was evaluated. As a result, compounds C and G, which possess small (acyloxy)alkyl ester, were unstable in buffers or medium (Fig. 9). In addition, compound L (with phenyl ester) was also unstable in buffers and medium, whereas compound O (with benzyl ester) was stable. It has been reported that ciprofloxacin analogues having an (acyloxy)alkyl ester are non-enzymatically degraded, and that increasing steric hindrance

around the (acyloxy)alkyl ester linker led to chemical stability (Zheng and Nolan, 2015). In addition, it has been reported that the rate of spontaneous hydrolysis of ferrocenylacrylate with phenyl ester was over 10-fold higher than that with benzyl ester (Menger and Ladika, 1987). The result of chemical stability in this study is consistent with these previous results showing that the compounds with (acyloxy)alkyl ester or phenyl ester have a potential to be degraded spontaneously. Thus, prodrugs having the (acyloxy)alkyl ester or phenyl ester should be required to be evaluated for spontaneous degradation. Among the compounds examined in this study, compound C, G, and L unlikely be selected as a prodrug with high priority.

Subsequently, the metabolic stability of the analogues and the formation rates of oseltamivir acid were evaluated using various human matrices (Fig. 10). Almost all the analogues were hydrolyzed to oseltamivir acid in only hepatocytes, as oseltamivir was. To select prodrugs that are efficiently and mainly converted to oseltamivir acid, the CS_{total} values were calculated by the metabolic stability assays (Table 15). Since compound D showed high metabolic stability, its CS_{total} value could not be calculated, suggesting that compound D would unlikely be selected as a candidate prodrug with high priority. The CS_{total} values of compounds S (0.4) and T (0.3) were prominently low, suggesting that these compounds would not be selected as candidate compounds because of the inefficient conversion into oseltamivir acid in the body.

Next, metabolic stabilities of the analogues and CS_{total} values using animal matrices were evaluated. In rat, the CS_{total} values of all test compounds were over 0.6. Although a previous study (Kisui et al., 2020) has reported that oseltamivir was hardly hydrolyzed in rat liver microsomes, the present study revealed that oseltamivir was hydrolyzed in rat hepatocytes. Since rat serum was added in the culture medium for rat hepatocytes in the present study, the oseltamivir hydrolysis in rat hepatocytes would be owing to enzyme(s) included in serum, like Ces1c. We added serum to hepatocytes, referring a paper reporting that *in vitro-in vivo* correlation of intrinsic clearance calculated from hepatocytes in the presence of serum was higher than those in the absence of serum (Shibata et al., 2000). It is

possible that the active metabolite formation might be overestimated if a target compound is hydrolyzed by enzymes in serum. In addition to compounds S and T, the CS_{total} values of compounds E, Q, V and W in dog (below 0.6) and those of compounds E, M, and Q in monkey (below 0.6) were lower than those in human. In Chapter 2, the cut-off value of the CS_{total} were set to 0.6, because the CR values in animal that were lower than in human were below 0.6. Since, in human, the analogues were hydrolyzed in the liver but not in small intestine, the appropriate animal for *in vivo* PK study for the analogues would be decided according to 1) the CS_{total} value and 2) the in small intestine metabolism in animal species. As shown in Table 16, the infeasibility of the intestinal metabolism of oseltamivir and compounds A, B, F, H, I, J, and K were common in the tested animals as well as human, suggesting that all tested animals could be used for their *in vivo* PK studies. In the case of compound M, the feature that it was not metabolized in intestine was common in human and tested animals, but the CS_{total} value was below 0.6 only in monkey; therefore, rat or dog can be used for *in vivo* PK study of compound M. Compounds N, P, and R were not metabolized in human, dog, and monkey small intestines, but were metabolized in rat. Thus, their *in vivo* PK studies should be conducted with dog and/or monkey. The infeasibility of the intestinal metabolism of compounds E and Q were common in the tested animals as well as human, and the CS_{total} values of them in rat were over 0.6, whereas those in dog and monkey were below 0.6. Therefore, rat should be used for their *in vivo* PK studies. Compounds O and U were not metabolized in human and dog small intestine, but they were metabolized in rat and monkey small intestine, suggesting that dog should be used for their *in vivo* PK studies. Compound V was not metabolized in human, dog, and monkey small intestines, whereas it was metabolized in rat small intestine; therefore monkey should be used for the *in vivo* PK study, because the CS_{total} value in dog was below 0.6. Thus, to select effective candidate compounds in humans, *in vitro* metabolism study using human and animal matrices as well as *in vivo* PK studies using animals selected according to the information of the *in vitro* study are necessary.

To examine the enzymes that catalyze the hydrolysis of oseltamivir and its analogues, their hydrolase activities by recombinant human CES1 and CES2 were evaluated (Fig. 11).

Table 16. Selection of animal species for *in vivo* PK study of oseltamivir analogues.

Test compounds	Rat		Dog		Monkey		Appropriate animal species
	CS_{total}	Similarity of intestinal metabolism to that in human matrices	CS_{total}	Similarity of intestinal metabolism to that in human matrices	CS_{total}	Similarity of intestinal metabolism to that in human matrices	
Oseltamivir	H	○	H	○	H	○	Rat, dog, monkey
Compound A	H	○	H	○	H	○	Rat, dog, monkey
Compound B	H	○	H	○	H	○	Rat, dog, monkey
Compound F	H	○	H	○	H	○	Rat, dog, monkey
Compound H	H	○	H	○	H	○	Rat, dog, monkey
Compound I	H	○	H	○	H	○	Rat, dog, monkey
Compound J	H	○	H	○	H	○	Rat, dog, monkey
Compound K	H	○	H	○	H	○	Rat, dog, monkey
Compound M	H	○	H	○	L	○	Rat, dog
Compound N	H	×	H	○	H	○	Dog, monkey
Compound P	H	×	H	○	H	○	Dog, monkey
Compound R	H	×	H	○	H	○	Dog, monkey
Compound E	H	○	L	○	L	○	Rat
Compound Q	H	○	L	○	L	○	Rat
Compound O	H	×	H	○	H	×	Dog
Compound U	H	×	H	○	H	×	Dog
Compound V	H	×	L	○	H	○	Monkey

The CS_{total} values of compounds listed here in human matrices were over 0.6.

H or L: The CS_{total} value was over or below 0.6.

○ or ×: The intestinal metabolism was similar or dissimilar to that in in human matrices.

Most of the analogues were hydrolyzed by CES1, and this result was consistent with the findings that they were hydrolyzed by human hepatocytes, in which CES1 is expressed, but not by human enterocytes in which CES1 is absent. We expected that the compounds having large sterically hindered cyclic structure (like compound T) or long chain normal alkane (like compounds U or W) would be hydrolyzed by CES2, because CES2 prefers compounds with a large alcohol group and small acyl group (Imai et al., 2006), whereas CES1 prefers compounds with a small alcohol group and large acyl group. However, these compounds were

not hydrolyzed by CES2. Imai et al (2006) have reported that propranolol derivatives having a branched acyl moiety with a methyl group at the 3-position were slightly hydrolyzed by CES2, whereas those with a methyl group at the 2-position were easily hydrolyzed. In some analogues used in Chapter 2, the alcohol group was enlarged considering the substrate preference of CES2, but such analogues were not hydrolyzed by CES2, probably because of the structural bulkiness. Thus, to intentionally create a compound that are hydrolyzed by CES2, the compound should be designed considering not only the size of alcohol group but also steric hindrance of the compounds.

In conclusion, this study could demonstrate, by using oseltamivir and its analogues, the usefulness of the systematic strategy for selecting prodrugs based on log D value, solubility in artificial intestine fluids, membrane permeability, and metabolic instability, which had been established in Chapter 1. It was demonstrated that the evaluation of metabolism using animal matrices enables the selection of appropriate animal species for *in vivo* PK study. I hope that this strategy would be used as one of tools to facilitate drug development.

CONCLUSION

Development of prodrugs is a useful strategy to overcome the defects of candidate drugs such as low bioavailability, short duration of action, and toxicity (Akhani et al., 2017; Huttunen et al., 2011). For certain active compounds, the concept of design of prodrugs has been published (Nofsinger et al., 2014; Schade et al., 2014), but general strategies for developing prodrugs with ideal pharmacokinetic properties remain to be established. Moreover, the selection of appropriate animals for pharmacokinetic and pharmacodynamics studies is important for prodrug development, because there are species differences in substrate specificities and tissue distributions of drug-metabolizing enzymes, including hydrolases, which catalyze the conversion to active metabolites from prodrugs (Fukami and Yokoi, 2012). This study aimed to establish a systematic *in vitro* method to appropriately select candidate prodrugs, which would contribute to increase the success rate in development of prodrugs.

In Chapter 1, physicochemical/biopharmaceutical properties (Log D, solubility, and membrane permeability) and metabolic stabilities of 21 model prodrugs with improved oral absorption and their active metabolites were evaluated. The log D values of prodrugs with improved membrane permeability were higher than those of their active metabolites, whereas those of prodrugs with improved aqueous solubility were lower than those of their active metabolites, indicating that the log D value would be a good index for the design of prodrug. As for the solubility, all prodrugs with improved aqueous solubility showed higher solubility in artificial intestinal fluids than their active metabolites, and artificial intestinal fluids can be likely used to evaluate the improvement of solubility of prodrugs. Evaluation of membrane permeability of prodrugs with improved membrane permeability by using Caco-2 cells, which are often used to predict the permeability of compounds in the human small intestine, revealed that membrane permeabilities of some prodrugs were lower than those of active metabolites. Thus, the membrane permeability assay using Caco-2 cells might not be suitable

for the selection of compounds for proceeding to *in vivo* PK study or development of a strategy for prodrugs. In addition, since the membrane permeability was correlated with the log D values, the measurement of log D might be sufficient for the evaluation of the membrane permeability to decide whether to proceed in development.

In metabolism study using human matrices, almost all the prodrugs were immediately hydrolyzed in enzymatic and/or non-enzymati manner, and some prodrugs were also hydrolyzed within intestinal epithelium cells. Therefore, it was suggested that the hydrolysis in the small intestine would not be the sole factor to exclude from candidate prodrugs. To examine the metabolic efficiency of the prodrugs, CS_{total} value, which represents the ratio of the apparent clearance for the active metabolite formation to the total clearance of prodrug, were defined. Prodrugs showed high CS_{total} values in human. In metabolism study using monkey and dog matrices, some prodrugs showed lower CS_{total} values than in human. CS_{total} values would reflect the metabolic profiles of each species, and might be a good parameter for selection of animals for *in vivo* PK studies to evaluate bioavailability showing similarity to humans.

Based on the physicochemical/biopharmaceutical properties and metabolic stabilities, a scheme for an *in vitro* screening of candidate prodrugs with sufficient oral absorption is depicted. In the 1st step, pharmacologically active compounds with low metabolic clearance are selected. In the 2nd step, the number of candidate prodrugs is narrowed down based on the criteria for log D, solubility in artificial intestinal fluids, membrane permeability, and human CS_{total}. In the 3rd step, animal species to be used in the subsequent *in vivo* PK studies is selected based on animal CS_{total}. In the 4th step, the *in vivo* PK in the selected experimental animal is evaluated. Based on the results from the *in vivo* PK analysis, in the 2nd step, and if necessary the 1st step, would be reapplied to select other candidate prodrugs. This scheme would streamline the development of prodrugs.

In Chapter 2, the utility of the systemic approach established in Chapter 1 was evaluated by using oseltamivir acid (active metabolite) and 23 kinds of its prodrugs with varied side chains, having varied physicochemical characteristics, as model compounds.

The log D values of the analogues were 2.0 to 4.9, and higher than that of oseltamivir acid (0.7). In the solubility, almost all the analogues showed high solubility (over 80%). In membrane permeability assay, PAMPA would be appropriate to evaluate the membrane permeability at least for oseltamivir analogues. In addition, since the P_e value was correlated with the log D value, the log D value would be a good index for the design of prodrug with improved membrane permeability. Almost all the analogues were hydrolyzed to oseltamivir acid only in human hepatocytes, as oseltamivir was. Moreover, the CS_{total} values of almost all analogues were over 0.6 in human. From the results of evaluation of metabolic stability using animal matrices, the appropriate animal for *in vivo* PK study for the analogues would be decided in two items: 1) CS_{total} value and 2) the in small intestine metabolism in animal species. Based on the physicochemical/biopharmaceutical properties and metabolic stabilities of oseltamivir and its analogues, compounds that are capable for proceeding to *in vivo* PK studies would be oseltamivir and 16 out of 23 analogues. In Chapter 2, it was verified for oseltamivir to be selected as one of the effective prodrugs by the strategy, and suitable experimental animals for *in vivo* PK studies would be different depending on candidate compounds.

In conclusion, this study successfully established a novel screening strategy for efficient selection of prodrug candidates and suitable experimental animals for *in vivo* PK studies. I hope this strategy would be useful for development of prodrugs that are converted to active metabolites by hydrolysis.

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ACKNOWLEDGEMENTS

I would like to express my deepest appreciation and sincere gratitude to Professor Miki Nakajima and Associate Professor Tatsuki Fukami for their constant guidance and encouragement in carrying out this research. I greatly appreciate Professors Ikumi Tamai, and Yukio Kato, and Associate Professor Yoshiyuki Shirasaka of Faculty of Pharmaceutical Sciences, Kanazawa University for valuable comments and suggestions as reviewers.

I am greatful to Mr. Hiroyuki Ogawa (Chemical Research Laboratories, Japan Tobacco Inc.) for synthesizing the analogues.

Finally, I would like to thank Mr. Yukihiro Nomura (Drug Metabolism and Pharmacokinetics Research Laboratories, Japan Tobacco Inc.) and Mr. Toshio Taniguchi (Drug Metabolism and Pharmacokinetics Research Laboratories, Japan Tobacco Inc.) for insightful comments and suggestions.

LIST OF PUBLICATIONS

Shimizu M, Fukami T, Taniguchi T, Nomura Y, and Nakajima M (2020) A novel systematic approach for selection of prodrugs designed to improve oral absorption. *J Pharm Sci* **109**: 1736-1746.