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Research Paper

Multichannel Liquid Chromatography–Tandem Mass Spectrometry Cocktail Method for Comprehensive Substrate Characterization of Multidrug Resistance-Associated Protein 4 Transporter

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Purpose. To develop a comprehensive substrate-screening method for the ATP-binding cassette (ABC) transporter, and identify new substrates for multidrug resistance-associated protein 4 (MRP4/ABCC4).
Methods. Human MRP4-expressing membrane vesicles were incubated with a mixture of 50 compounds, including methotrexate, a known MRP4 substrate. The amounts transported were simultaneously determined by liquid chromatography–tandem mass spectrometry.
Results. From 49 compounds, 12 were identified as substrate candidates for MRP4 in the first screening. The second screening was performed involving the uptake of mixture using single quadrupole multichannel mode, and the third screening was performed involving the uptake of individual compounds using multiple reaction monitoring multichannel mode. As a result, eight substrate candidates were additionally identified. Subsequently, in the fourth step, osmotic pressure-dependent transport was demonstrated for 18 compounds (cefmetazole, piperacillin, rebamipide, tetracycline, ampicillin, benzylpenicillin, bumetanide, cephalosporin C, enalapril, pipemidic acid, furosemide, ceftazidime, pravastatin, hydrochlorothiazide, sulbactam, baclofen, bezafibrate and alacepril) among the 20 substrate candidates, thereby confirming them as MRP4 substrates. By contrast, the uptakes of meloxicam and nateglinide did not depend on osmolarity, indicating that these compounds were not substrates, but bound to MRP4.
Conclusions. The new comprehensive substrate-screening method for ABC transporters allowed the identification of 18 new substrates for MRP4.

KEY WORDS: ATP-binding cassette transporter; human multidrug resistance-associated protein 4; liquid chromatography–tandem mass spectrometry; substrate screening; vesicle uptake study.

INTRODUCTION

The human multidrug resistance-associated protein 4 (MRP4/ABCC4) is a subtype of the ABC transporter superfamily, and it excretes substrates from the inside to the outside of cells by coupling with ATP hydrolysis. MRP4 is expressed in a number of organs, including the kidney and brain, and contributes to the distribution and elimination of predominantly organic anions (1). In the kidney, MRP4 is localized at the proximal tubule apical membrane, and is involved in tubular secretion into the lumen (2,3). The messenger RNA (mRNA) level of MRP4 in the kidney is the highest among all of the transporters that contribute to the apical efflux transport of organic anions in the proximal tubules, such as MRP2, MRP4, organic anion transporter 4 (OAT4), and ABC transporter G2 (ABCG2) (4). Furthermore, MRP4 plays an important role in restricting the distribution of drugs in the central nervous system (CNS). An *in vivo* study has demonstrated that MRP4, which is localized at the luminal membrane of the brain capillary endothelium and at the basolateral membrane of the choroid plexus epithelium, restricts penetration of the anticancer agent topotecan into the brain and cerebrospinal fluid (CSF) (5).

β-Lactam antibiotics, diuretics, non steroidal anti-inflammatory drugs (NSAIDs), and angiotensin-converting enzyme (ACE) inhibitors are among the most important groups of organic anionic drugs undergoing tubular secretion (6–9). Additionally, it has been reported that anionic drugs, such as β-lactam antibiotics, the 5-hydroxy-3-methyl-

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ABBREVIATIONS: ABC, ATP-binding cassette; ACE, angiotensin-converting enzyme; BBB, blood–brain barrier; BCSFB, blood–cerebrospinal fluid barrier; CMZ, cefmetazole; CNS, central nervous system; DHEAS, dehydroepiandrosterone sulfate; ESI, electrospray ionization; LC–MS, liquid chromatography–mass spectrometry; LC–MS/MS, liquid chromatography–tandem mass spectrometry; MRM, multiple reaction monitoring; MRP, multidrug resistance-associated protein; MTX, methotrexate; MUI_D, membrane uptake-index difference; OAT, organic anion transporter; Q1, single quadrupole.

glutaryl-coenzyme A (HMG-CoA) reductase inhibitor pravastatin, centrally acting anionic NSAIDs, and baclofen, have poor brain distributions (10–14). However, little is known about whether MRP4 is involved in the renal tubular secretion and/or the restricted distribution in the CNS of these various anionic drugs. To clarify whether MRP4 is involved in such processes, a comprehensive substrate screening procedure needs to be applied to these drugs.

Substrate screening for MRP4 has been performed by means of vesicle uptake studies, cell efflux transport assays, cytotoxicity assays, ATPase assays, and inhibition assays against vesicle uptake of a known substrate, and also by means of *in vivo* studies using knockout mice (5,15–17). Among these assays, vesicle-uptake study only allows evaluation of the direct transport of a compound via MRP4. Therefore, vesicle-uptake study is suitable for the precise identification of substrates. However, the majority of test compounds have been limited to labeled compounds, and uptake studies are time-consuming, thereby preventing comprehensive substrate screening by means of vesicle-uptake studies.

Liquid chromatography–mass spectrometry (LC–MS) is one of the most widely used analytical techniques for non-labeled small compounds. LC–MS has become an essential methodology for drug metabolism and pharmacokinetic screening, including cytochrome P450 enzyme-inhibition screening and Caco-2-absorption screening (18). Recent advances in MS have allowed the simultaneous measurement of >50 molecules (19). Furthermore, by applying LC–tandem MS (MS/MS), analytes can be measured with a high sensitivity, comparable to that achieved using radioisotopes. Therefore, by taking advantage of this analytical technique, a comprehensive substrate screening based on vesicle uptake can be performed.

The purpose of the present study was to develop a comprehensive and rapid substrate-screening method for MRP4 by employing both vesicle-uptake study and LC–MS/MS, and to establish whether 49 compounds were in fact substrates of MRP4.

MATERIALS AND METHODS (LC–MS/MS COCKTAIL METHOD)

Reagents

Amoxicillin, azathioprine, (±)-baclofen, bezafibrate, bumetanide, caffeine, captopril, cefmetazole sodium salt (CMZ), ceftazidime hydrate, cephalosporin C zinc salt, dantrolene sodium salt, dehydroepiandrosterone sulfate (DHEAS), enalapril maleate salt, enoxacin, furosemide, hydrochlorothiazide, ketoprofen, meclofenamic-acid sodium salt, nalidixic acid, neostigmine bromide, norfloxacin, ofloxacin, pipemidic acid, piroxicam, salicylic acid, sulindac, tetracycline, theobromine, *trans*-4-(aminomethyl)cyclohexanecarboxylic acid (tranexamic acid), and warfarin were purchased from Sigma Chemical Co. (St. Louis, MO). Alacepril, allopurinol, ampicillin sodium, diclofenac sodium, ethacrynic acid, indomethacin, mefenamic acid, meloxicam, methotrexate (MTX), pravastatin sodium salt, sulbactam sodium salt, and sulpyrine monohydrate were purchased from Wako Pure Chemicals (Osaka, Japan). Ciprofloxacin hydrochloride, L-glutamine, and benzylpenicillin sodium salt were purchased from ICN Biomedicals Inc. (Aurora, OH). Alendronate, potassium canrenoate, and rebamipide were purchased from LKT Laboratories Inc. (St. Paul, MN). L-glutamic acid and theophylline were purchased from Nacalai Tesque Inc. (Kyoto, Japan). Isosorbide dinitrate was purchased from Alexis Biochemicals (San Diego, CA). Nateglinide was purchased from Toronto Research Chemicals Inc. (North York, Canada). Nicorandil was purchased from Tocris Bioscience (Ellisville, MO). Piperacillin sodium salt was purchased from MP Biomedicals Inc. (Eschwege, Germany). Control vesicles and human MRP4-expressing membrane vesicles were purchased from GenoMembrane (Yokohama, Japan) or were kindly supplied by GenoMembrane. All other chemicals were of reagent grade, and were available commercially.

Rapid and Conventional Substrate Screening by Single Quadrupole (Q1) Multichannel LC–MS Analysis Following a Cocktail Transport Study of Membrane Vesicles

1. Simultaneous vesicle-uptake study to determine the MRP4 transport rate for a cocktail of compounds

Uptake experiments were performed using the rapid-filtration method, as described previously (20), and these were carried out in medium containing membrane vesicles, 0.25 M sucrose, 10 mM Tris–HCl (pH 7.4), 10 mM $MgCl_2$, 4 mM ATP, 10 mM phosphocreatine, 100 μg/ml creatine phosphokinase, a mixture of test compounds, and MTX, which is a known substrate for MRP4 (21), in a total volume of 220 μl. The reactions were carried out at 37°C and stopped by the addition of 700 μl ice-cold stop solution (0.25 M sucrose, 10 mM Tris–HCl (pH 7.4), and 100 mM NaCl). The samples were passed through 0.22 μm Durapore membrane filters (Millipore, Bedford, MA) under vacuum. The filters were then washed three times with 3 ml ice-cold stop solution, and extracted with 1 ml methanol. The extracts were concentrated to 50 μl by centrifugation under vacuum. Then, 150 μl 0.1% formic acid in water was added to 50 μl of the concentrated extracts. A sample of 100 μl was used for the LC–MS quantification, as described below.

2. Simultaneous quantification of intravesicular uptake for multiple compounds by Q1 multichannel LC–MS

The sample analysis was automated by coupling a triple quadrupole mass spectrometer (4000QTRAP, Applied Biosystems) to an Agilent 1100 high-performance liquid chromatography (HPLC) system (Agilent Technologies). Briefly, a 100 μl uptake sample was injected onto a reversed-phase HPLC column (XDB-C18; 2.1×150 mm; particle size 5 μm; Agilent). Mobile phases A and B consisted of 0.1% formic acid in water and 0.1% formic acid in acetonitrile, respectively. In the first screening, the compounds were separated and eluted from the column using a linear gradient with a 110-min run time at a flow rate of 0.2 ml/min, and the sequence was as follows: A–B (100:0) for 5 min after injection, 0:100 at 65 min and up to 75 min, 100:0 at 85 min and up to 110 min. In the second screening, the compounds were separated and eluted from the column using a linear

gradient with a 60-min run time at a flow rate of 0.2 ml/min, and the sequence was as follows: A–B (100:0) for 5 min after injection, 0:100 at 30 min and up to 32 min, 100:0 at 35 min and up to 60 min. The ion source was set as the electrospray ionization (ESI) positive-ionization mode. The MS operating conditions were as follows: curtain gas, 40; ionspray voltage, 5,000 V; temperature, 600°C; ion source gas 1, 50 p.s.i.; ion source gas 2, 80 p.s.i.; and declustering potential, 60 V. The compounds were simultaneously and selectively determined using 50 *m/z* channels, which were specific for each compound, in the Q1 multiple ions mode (Table I).

3. Evaluation of the MRP4-dependent transport rate

The uptake was calculated from the peak area on the mass chromatogram, and was expressed as the vesicle-to-medium ratio, which was obtained by dividing the amount taken up into the membrane vesicles by the substrate concentration in the uptake medium. To evaluate the MRP4-dependent transport rate, the membrane uptake-index difference (MUI_D) of each compound was calculated from the following equation:

$$MUI_D = vesicle/medium\ ratio_{MRP4} - vesicle/medium\ ratio_{Mock.}$$

Here, the vesicle-to-medium $ratio_{MRP4}$ and the vesicle-to-medium $ratio_{Mock}$ are the uptake of compounds into membrane vesicles prepared from MRP4-transfected and parental vector-transfected Sf9 cells, respectively.

Highly Sensitive Substrate Screening by MRM Multichannel LC–MS/MS Analysis Following an Individual Transport Study or Osmotic Pressure Dependency Study

1. Individual vesicle-uptake study to determine the MRP4 transport rate for a single compound

Uptake experiments were performed as described above, and were carried out with a single test compound in the medium containing membrane vesicles, 0.25 M sucrose, 10 mM Tris–HCl (pH 7.4), 10 mM $MgCl_2$, 4 mM ATP, 10 mM phosphocreatine, 100 μg/ml creatine phosphokinase, and the test compound, in a total volume of 110 μl. The reactions were carried out at 37°C, and were stopped by the addition of 800 μl ice-cold stop solution. The samples were then passed through 0.22 μm Durapore membrane filters under vacuum. The filters were washed three times with 3 ml ice-cold stop solution, and were extracted with 1 ml methanol. The extracts were concentrated to 50 μl by centrifugation under vacuum. Then, 150 μl 0.1% formic acid in water was added to 50 μl of the concentrated extracts. Next, 200 μl samples of several test compounds were mixed. An aliquot of 100 μl was used for the LC–MS/MS quantification, as described below.

2. Simultaneous quantification of intravesicular uptake for several compounds by MRM multichannel LC–MS/MS

A 100-μL uptake sample mixture of several test compounds was injected onto a reversed-phase HPLC column (XDB-C18; 2.1×150 mm; particle size 5 μm). The compounds were separated and eluted from the column using a linear gradient with a 30-min run time at a flow rate of 0.2 ml/min, and the sequence was as follows: A–B (100:0) for 5 min after injection, 0:100 at 15 min, 100:0 at 16 min and up to 30 min. The eluted compounds were simultaneously and selectively determined by using the multiplexed MRM channels in the ESI positive or negative ionization mode. Each MRM channel was specific for a compound, as listed in Table I. The conditions of the MRM channel were optimized using automatic optimization software (Analyst, ABI) by the direct infusion of 1–100 μM compound solution at a flow rate of 5 μl/min with a syringe pump (Harvard) into 4000QTRAP. The uptake was expressed as the vesicle-to-medium ratio, and the MUI_D was determined as described above.

Data Analysis

For kinetic studies, the K_m and V_{max} of CMZ by MRP4-expressing membrane vesicles were estimated from the following equation, using the nonlinear least-squares regression analysis program MULTI (22):

$$v = V_{max} \times [S]/(K_m + [S]).$$

Here, v and $[S]$ are the uptake rate and the concentration of CMZ, respectively.

Unless otherwise indicated, all data represent the mean ± standard error of the mean (SEM). An unpaired, two-tailed, Student's t test was used to determine the significance of differences between the means of two groups. One-way analysis of variance (ANOVA) followed by the modified Fisher's least-squares difference method was used to assess the statistical significance of differences among the means of more than two groups.

RESULTS

Scheme 1 presents a flow chart of the LC–MS/MS cocktail method, which allows the rapid and comprehensive screening and identification of MRP4 substrates by means of conventional Q1 multichannel LC–MS analysis following cocktail transport studies, and then highly-sensitive MRM multichannel LC–MS/MS analysis following individual transport studies or osmotic pressure-dependency studies. The substrate/non-substrate classification was established for all of the compounds by four steps of independent transport studies. The 49 test compounds selected were mainly organic anions, including those known to be extensively excreted from the kidney and/or to have restricted distribution into the brain, but not previously reported as substrates of MRP4.

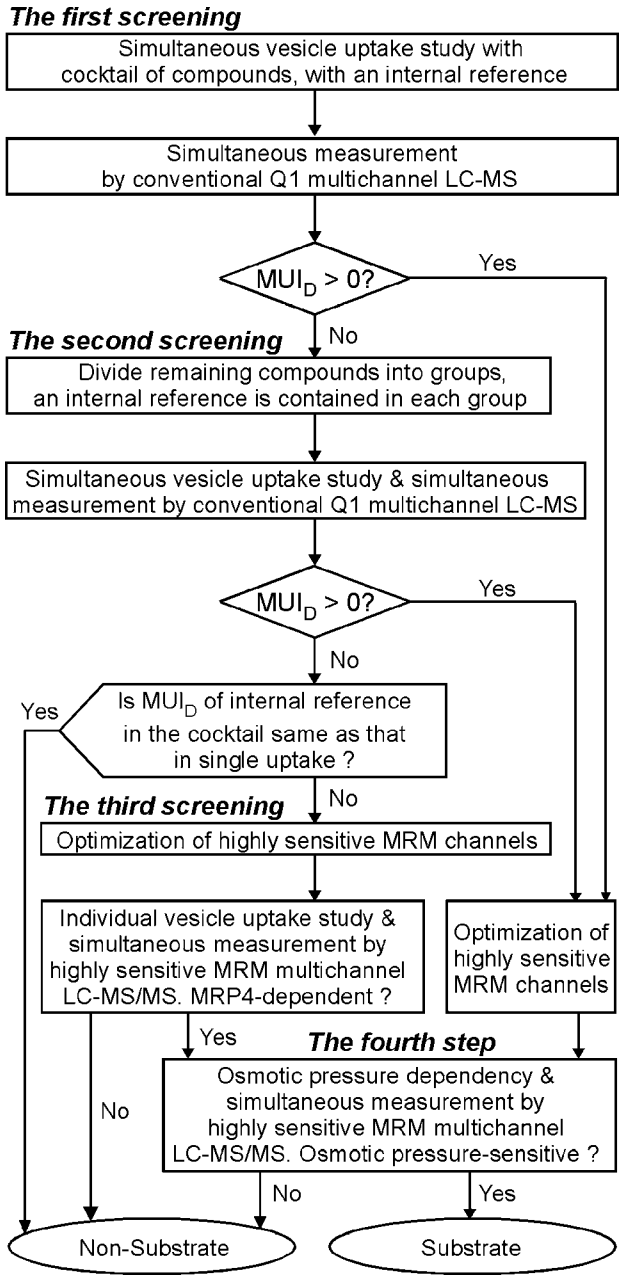
The First Screening; Rapid and Conventional Substrate Screening by Q1 Multichannel LC–MS Analysis Following a Cocktail Transport Study

The *m/z* channels of all compounds examined, including MTX, are listed in Table I, and were simultaneously analyzed by Q1 multichannel LC–MS. MTX was used as an internal

Table I. *m/z* channels of 50 compounds in Q1 multichannel LC–MS and MRM channels of several compounds in MRM multichannel LC–MS/MS

Compound	Monoisotopic mass	*m/z* channel	MRM channels	Compound	Monoisotopic mass	*m/z* channel	MRM channels
Allopurinol	136.03	137.03		Nateglinide	317.20	318.20	318.3/165.8
Salicylate	138.03	139.03	139.1/79.1	Norfloxacin	319.13	320.13	
Glutamine	146.07	147.07		Enoxacin	320.13	321.13	
Glutamic acid	147.05	148.05		Furosemide	330.01	331.01	328.9/205.0
Tranexamic acid	157.11	158.11		Piroxicam	331.06	332.06	
Theobromine	180.06	181.06		Ciprofloxacin	331.13	332.13	
Theophylline	180.06	181.06		Benzylpenicillin	334.10	335.10	335.2/91.1
Caffeine	194.08	195.08		Ampicillin	349.11	350.11	350.2/106.1
Nicorandil	211.06	212.06		Meloxicam	351.03	352.03	352.0/115.1
Baclofen	213.05	214.05	214.1/151.1	Sulindac	356.08	357.08	357.2/233.2
Captopril	217.08	218.08	218.2/56.1	Canrenoate	358.21	359.21	
Neostigmine[a]	223.14	223.14		Bezafibrate	361.11	362.11	362.1/139.1
Nalidixic acid	232.08	233.08	233.2/104.1	Ofloxacin	361.14	362.14	
Sulbactam	233.04	234.04	232.2/140.1	Bumetanide	364.11	365.11	365.1/240.1
Isosorbide dinitrate	236.03	237.03		Amoxicillin	365.10	366.10	366.1/114.1
Mefenamic acid	241.11	242.11		Rebamipide	370.07	371.07	371.0/216.0
Alendronate	249.02	250.02		Enalapril	376.20	377.20	377.2/234.1
Azathioprine	277.04	278.04	278.0/142.0	Alacepril	406.15	407.15	407.2/242.1
Meclofenamic acid	295.02	296.02		Cephalosporin C	415.10	416.10	416.1/312.1
Hydrochlorothiazide	296.96	297.96	295.9/268.9	Pravastatin	424.25	425.25	423.2/321.2
Ethacrynic acid	302.01	303.01	303.1/257.0	Tetracycline	444.15	445.15	445.2/410.1
Pipemidic acid	303.13	304.13	304.1/217.1	MTX	454.17	455.17	455.2/308.0
Warfarin	308.10	309.10		CMZ	471.05	472.05	472.0/356.1
Sulpyrine	311.09	312.09	312.1/77.1	Piperacillin	517.16	518.16	518.1/142.9
Dantrolene	314.07	315.07	313.0/227.9	Ceftazidime	546.10	547.10	547.1/468.2

The *m/z* channels were set at the monoisotopic masses plus one. The MRM channels were obtained by direct infusion of the compound solution into 4000QTRAP.
[a] *m/z* channel of neostigmine was assumed to have the same value as the monoisotopic mass, since the compound is a quaternary amine.



Scheme 1. Strategy for rapid and comprehensive substrate and non-substrate screening for MRP4 using the LC–MS/MS cocktail method. The internal reference was MTX, a known substrate of MRP4

reference to evaluate the inhibitory effect of compounds on MRP4-transport activity. The first screening was performed twice, primary and secondary screenings. The primary first screening was performed on a mixture of 50 compounds (Table I) at a concentration of 10 μM in 100 μg membrane vesicles prepared from MRP4-transfected Sf9 cells for 30 min at 37°C. At this concentration, the inhibitory effect of MTX on substrate transport via MRP4 would be negligible, because the K_m value of MTX is 220 μM. Membrane vesicles prepared from the parent vector-transfected Sf9 cells were used for a control study in the same manner. Table II summarizes the results, listing the vesicle/medium $ratio_{MRP4}$ and the vesicle/medium $ratio_{Mock}$ as the apparent transport rate into MRP4-expressing membrane vesicles and into the control membrane vesicles, respectively. The apparent MRP4-dependent transport rate was determined for each compound by subtracting the value of the vesicle/medium $ratio_{Mock}$ from that of the vesicle/medium $ratio_{MRP4}$, and is listed as the MUI_D in Table II. A significant apparent MRP4-dependent transport rate was obtained for CMZ, piperacillin, MTX, rebamipide, tetracycline, ampicillin, benzylpenicillin, and bumetanide (Table II). In the absence of the compounds listed in Table II, the MRP4-dependent transport rate of MTX was determined as 38.4 ± 1.0 μl/(mg protein·30 min) (Table III). A significant inhibition (by 85.5%) in the presence of the 49 compounds (5.55 ± 0.27 μl/(mg protein·30 min)) suggested that the MRP4-dependent transport rate of the tested compounds was underestimated due to mutual inhibition (Table II). As isosorbide dinitrate, allopurinol, alendronate, and glutamine were not detected at 10 pmol by LC–MS, we were unable to determine their transport rates. Hydrochlorothiazide was not detected by LC–MS in the MRP4-expressing and control membrane vesicles, though it was detected at the 10 pmol level by LC–MS.

The secondary first screening was performed for a mixture of 39 compounds, excluding seven substrate candidates and four undetectable compounds from the list shown in Table II, and the results are summarized in Table III. A significant apparent MRP4-dependent transport rate was obtained for MTX, cephalosporin C, enalapril, nateglinide, pipemidic acid, and meloxicam (Table III). The MTX-transport rate was 6.48 ± 0.51 μl/(mg protein·30 min) in the presence of the 38 compounds, which was 16.1% of the control value, again suggesting an underestimation of the MRP4-transport rate for all of the compounds (Table III).

The Second Screening; Substrate Screening of Compounds Divided Into 4 Groups by Q1 Multichannel LC–MS Analysis Following a Cocktail Transport Study

The compounds that failed to demonstrate significant apparent MRP4-dependent transport in Table III were divided into four groups as shown in Table IV. The second substrate screening was performed for each group using a 5 μM mixture with MTX for 60 min. No significant MRP4-dependent transport rate (MUI_D) was observed for any compound examined, except MTX (Table IV). No significant inhibitory effect on the MTX-transport rate was obtained for groups 3 and 4, confirming that the compounds in these groups are not effective MRP4 substrates. In contrast, in groups 1 and 2, there was a significant reduction in the MTX transport rate to 12.7 and 21.9% of that of the control study (60.7 ± 1.1 μl/(mg protein·60 min) without mixture), respectively (Table IV). Therefore, the possibility cannot be ruled out that a significant inhibitory effect was causing an underestimation of the MRP4-dependent transport activity in these groups.

The Third Screening; Highly Sensitive Substrate Screening by MRM Multichannel LC–MS/MS Analysis Following an Individual Transport Study

A third substrate screening was performed by individual transport studies of the compounds in groups 1 and 2 shown in Table IV, and the results are summarized in Table V. After an individual transport study, the extracts of 12

Table II. MRP4-dependent uptake of 50 compounds into membrane vesicles in the primary first screening

Compound	Vesicle/medium ratio (μl/(mg protein·30 min))		MUI_D μl/(mg protein·30 min)
	MRP4	Mock	
MTX	6.93 ± 0.27	1.37 ± 0.05	5.55 ± 0.27***
CMZ	7.06 ± 0.14	0.374 ± 0.027	6.68 ± 0.14***
Piperacillin	6.55 ± 0.38	0.669 ± 0.019	5.88 ± 0.38***
Rebamipide	7.52 ± 0.05	3.64 ± 0.10	3.88 ± 0.05***
Tetracycline	17.3 ± 0.8	15.7 ± 0.5	1.55 ± 0.81***
Ampicillin	1.99 ± 0.06	0.556 ± 0.039	1.44 ± 0.06***
Benzylpenicillin	1.69 ± 0.23	0.314 ± 0.053	1.37 ± 0.23**
Bumetanide	3.66 ± 0.21	2.50 ± 0.20	1.17 ± 0.21*
Ceftazidime	2.72 ± 0.17	1.90 ± 0.05	0.823 ± 0.175
Canrenoate	0.908 ± 0.102	0.103 ± 0.005	0.806 ± 0.102
Cephalosporin C	1.69 ± 0.01	0.942 ± 0.146	0.744 ± 0.013
Alacepril	1.07 ± 0.01	0.461 ± 0.031	0.613 ± 0.006
Amoxicillin	1.84 ± 0.20	1.25 ± 0.29	0.589 ± 0.199
Sulbactam	0.548 ± 0.050	0.0768 ± 0.0088	0.471 ± 0.050
Nalidixic acid	1.65 ± 0.04	1.18 ± 0.04	0.468 ± 0.042
Enalapril	0.785 ± 0.025	0.311 ± 0.029	0.474 ± 0.025
Mefenamic acid	1.83 ± 0.13	1.49 ± 0.04	0.343 ± 0.135
Captopril	1.04 ± 0.05	0.764 ± 0.059	0.276 ± 0.052
Baclofen	1.01 ± 0.02	0.758 ± 0.057	0.250 ± 0.020
Pipemidic acid	0.620 ± 0.014	0.404 ± 0.026	0.217 ± 0.014
Norfloxacin	0.536 ± 0.018	0.409 ± 0.040	0.127 ± 0.018
Bezafibrate	0.345 ± 0.019	0.244 ± 0.033	0.101 ± 0.019
Furosemide	3.28 ± 0.25	3.21 ± 0.38	0.0649 ± 0.2466
Piroxicam	1.38 ± 0.03	1.32 ± 0.02	0.0607 ± 0.0326
Sulindac	0.355 ± 0.031	0.319 ± 0.018	0.0361 ± 0.0309
Ciprofloxacin	0.358 ± 0.037	0.328 ± 0.038	0.0299 ± 0.0367
Enoxacin	0.465 ± 0.030	0.437 ± 0.032	0.0281 ± 0.0297
Ofloxacin	0.160 ± 0.009	0.176 ± 0.032	−0.0167 ± 0.0087
Nateglinide	0.630 ± 0.025	0.657 ± 0.065	−0.0273 ± 0.0250
Theobromine	0.502 ± 0.116	0.544 ± 0.005	−0.0419 ± 0.1154
Warfarin	0.675 ± 0.058	0.734 ± 0.031	−0.0594 ± 0.0584
Caffeine	0.732 ± 0.017	0.833 ± 0.075	−0.101 ± 0.017
Azathioprine	0.0844 ± 0.0093	0.194 ± 0.020	−0.110 ± 0.009
Ethacrynic acid	1.47 ± 0.05	1.65 ± 0.06	−0.183 ± 0.052
Pravastatin	3.61 ± 0.55	3.95 ± 0.46	−0.332 ± 0.553
Tranexamic acid	2.07 ± 0.18	2.48 ± 0.11	−0.407 ± 0.177
Meloxicam	3.75 ± 0.09	4.24 ± 0.06	−0.487 ± 0.086
Theophylline	0.184 ± 0.036	0.746 ± 0.038	−0.562 ± 0.036
Dantrolene	1.25 ± 0.05	2.26 ± 0.16	−1.00 ± 0.06
Salicylate	3.15 ± 0.22	4.61 ± 0.76	−1.46 ± 0.22
Meclofenamic acid	5.54 ± 0.56	7.58 ± 0.24	−2.05 ± 0.56
Neostigmine	3.17 ± 0.17	5.51 ± 0.07	−2.34 ± 0.17
Sulpyrine	4.04 ± 0.30	6.58 ± 0.30	−2.54 ± 0.30
Glutamic acid	2.32 ± 0.36	5.47 ± 0.07	−3.15 ± 0.36
Nicorandil	0.230 ± 0.032	4.08 ± 0.58	−3.86 ± 0.03
Isosorbide dinitrate[a]	–	–	–
Allopurinol[a]	–	–	–
Alendronate[a]	–	–	–
Glutamine[a]	–	–	–
Hydrochlorothiazide[b]	–	–	–

Membrane vesicles (100 μg) prepared from MRP4-transfected or parental vector-transfected Sf9 cells (Mock) were incubated at 37°C for 30 min in uptake medium containing 50 compounds (each 10 μM) in the presence of 4 mM ATP. Each value represents the mean ± SEM (n=4). *p<0.05, **p<0.01, ***p<0.001, significantly greater than 0 μl/(mg protein·30 min) of MUI_D, Dunnett's test.

[a] The authentic samples (10 pmol) were not detected by LC–MS, and the compounds were also not detected in uptake samples of membrane vesicles prepared from MRP4-transfected and parental vector-transfected Sf9 cells.

[b] The authentic sample (10 pmol) was detected, but the compound was not detected in uptake samples.

Table III. MRP4-dependent uptake of 39 compounds into membrane vesicles in the secondary first screening

Compound	Vesicle/medium ratio (μl/(mg protein·30 min))		MUI_D μl/(mg protein·30 min)
	MRP4	Mock	
MTX (single)	39.6 ± 1.0	1.13 ± 0.04	38.4 ± 1.0
MTX	7.44 ± 0.51	0.963 ± 0.243	6.48 ± 0.51***,†††
Cephalosporin C	1.93 ± 0.12	0.561 ± 0.145	1.37 ± 0.12***
Enalapril	1.16 ± 0.03	0.139 ± 0.005	1.02 ± 0.03***
Nateglinide	1.51 ± 0.08	0.548 ± 0.061	0.964 ± 0.083**
Pipemidic acid	1.14 ± 0.06	0.321 ± 0.099	0.818 ± 0.058*
Meloxicam	3.25 ± 0.10	2.43 ± 0.42	0.816 ± 0.097*
Sulpyrine	6.17 ± 0.13	5.50 ± 0.89	0.670 ± 0.135
Ciprofloxacin	0.864 ± 0.056	0.251 ± 0.037	0.612 ± 0.056
Alacepril	1.12 ± 0.02	0.507 ± 0.050	0.609 ± 0.021
Norfloxacin	0.920 ± 0.063	0.323 ± 0.107	0.597 ± 0.063
Bezafibrate	0.729 ± 0.030	0.212 ± 0.029	0.517 ± 0.030
Baclofen	0.846 ± 0.025	0.336 ± 0.063	0.509 ± 0.025
Enoxacin	0.845 ± 0.053	0.352 ± 0.108	0.493 ± 0.053
Ethacrynic acid	1.65 ± 0.06	1.16 ± 0.09	0.491 ± 0.062
Dantrolene	1.37 ± 0.06	0.909 ± 0.088	0.465 ± 0.064
Piroxicam	1.38 ± 0.05	0.919 ± 0.141	0.465 ± 0.050
Tranexamic acid	1.39 ± 0.19	1.01 ± 0.25	0.384 ± 0.191
Canrenoate	1.62 ± 0.19	1.26 ± 0.22	0.361 ± 0.187
Warfarin	0.816 ± 0.016	0.561 ± 0.036	0.255 ± 0.016
Nalidixic acid	0.475 ± 0.023	0.299 ± 0.076	0.176 ± 0.023
Glutamic acid	1.02 ± 0.19	0.860 ± 0.311	0.160 ± 0.190
Caffeine	0.867 ± 0.069	0.711 ± 0.116	0.156 ± 0.069
Neostigmine	1.52 ± 0.16	1.38 ± 0.37	0.138 ± 0.159
Sulindac	0.375 ± 0.007	0.240 ± 0.012	0.135 ± 0.007
Captopril	0.809 ± 0.182	0.686 ± 0.177	0.123 ± 0.182
Salicylate	3.18 ± 0.26	3.09 ± 0.11	0.0900 ± 0.2612
Ofloxacin	0.144 ± 0.014	0.103 ± 0.008	0.0412 ± 0.0139
Theophylline	0.409 ± 0.104	0.402 ± 0.124	0.00715 ± 0.10374
Azathioprine	0.370 ± 0.067	0.382 ± 0.111	−0.0115 ± 0.0669
Amoxicillin	1.85 ± 0.25	1.96 ± 0.16	−0.109 ± 0.246
Sulbactam	0.685 ± 0.093	0.799 ± 0.175	−0.114 ± 0.093
Mefenamic acid	1.68 ± 0.14	1.80 ± 0.13	−0.123 ± 0.145
Furosemide	2.29 ± 0.15	2.44 ± 0.15	−0.141 ± 0.146
Nicorandil	0.724 ± 0.078	0.879 ± 0.062	−0.156 ± 0.078
Pravastatin	3.01 ± 0.24	3.18 ± 0.47	−0.171 ± 0.240
Theobromine	0.523 ± 0.104	0.720 ± 0.089	−0.198 ± 0.104
Ceftazidime	2.41 ± 0.16	3.03 ± 0.69	−0.630 ± 0.161
Meclofenamic acid	5.22 ± 0.44	6.94 ± 0.53	−1.72 ± 0.44
Hydrochlorothiazide[a]	–	–	–

Membrane vesicles (100 μg) prepared from MRP4-transfected or parental vector-transfected Sf9 cells (Mock) were incubated at 37°C for 30 min in uptake medium containing 39 compounds (each 10 μM) or 10 μM MTX only in the presence of 4 mM ATP. Each value represents the mean ± SEM ($n = 4$).

*$p<0.05$, **$p<0.01$, ***$p<0.001$, significantly greater than 0 μl/(mg protein·30 min) of MUI_D, Dunnett's test.

††† $p<0.001$, significantly different from MUI_D of MTX (single), Student's t test.

[a] The authentic (10 pmol) sample was detected by LC–MS, while the compound was not detected in uptake samples of membrane vesicles prepared from MRP4-transfected and parental vector-transfected Sf9 cells.

(positively ionized) or five (negatively ionized) compounds from membrane vesicles were combined, which allowed us to shorten the analysis time, and the intravesicular amounts of the respective compounds were simultaneously determined by highly sensitive LC–MS/MS in the MRM multichannel mode. A significant apparent MRP4-dependent transport was seen using furosemide, ceftazidime, pravastatin, hydrochlorothiazide, sulbactam, baclofen, bezafibrate, and alacepril, while no significant MRP4-dependent transport was obtained for dantrolene, amoxicillin, sulpyrine, ethacrynic acid, azathioprine, sulindac, captopril, salicylate, or nalidixic acid.

The Fourth Step; Precise Identification of MRP4 Substrates by MRM Multichannel LC–MS/MS Analysis Following an Osmotic Pressure Dependency Study

To clarify whether the apparent MRP4-dependent transport represented transport via MRP4 or binding to MRP4, the

Table IV. MRP4-dependent uptake of 34 compounds in four groups in the second screening

	Compound	Vesicle/medium ratio (μl/(mg protein·60 min))		MUI_D μl/(mg protein·60 min)
		MRP4	Mock	
	MTX (single)	64.3 ± 1.1	3.62 ± 0.38	60.7 ± 1.1
	MTX	12.3 ± 0.7	4.62 ± 0.32	7.68 ± 0.66*,**
	Ethacrynic acid	1.06 ± 0.16	0.807 ± 0.162	0.252 ± 0.160
	Baclofen	2.17 ± 0.12	1.93 ± 0.24	0.241 ± 0.120
	Azathioprine	0.458 ± 0.127	0.550 ± 0.145	−0.0928 ± 0.1273
1	Bezafibrate	1.42 ± 0.13	1.56 ± 0.39	−0.143 ± 0.130
	Sulbactam	0.937 ± 0.011	1.12 ± 0.30	−0.187 ± 0.011
	Dantrolene	1.46 ± 0.09	1.84 ± 0.23	−0.384 ± 0.090
	Sulpyrine	7.00 ± 0.40	7.44 ± 0.48	−0.446 ± 0.404
	Nalidixic acid	2.70 ± 0.12	3.89 ± 0.84	−1.19 ± 0.12
	MTX	18.6 ± 0.9	5.30 ± 0.30	13.3 ± 0.9*,**
	Ceftazidime	3.33 ± 0.08	2.56 ± 0.32	0.772 ± 0.083
	Furosemide	5.41 ± 0.28	4.70 ± 0.25	0.713 ± 0.282
	Alacepril	2.53 ± 0.09	2.01 ± 0.19	0.522 ± 0.088
2	Amoxicillin	3.23 ± 0.10	2.76 ± 0.60	0.474 ± 0.101
	Pravastatin	5.78 ± 0.35	5.50 ± 1.00	0.280 ± 0.352
	Sulindac	1.79 ± 0.12	1.69 ± 0.12	0.102 ± 0.118
	Captopril	0.630 ± 0.038	0.683 ± 0.040	−0.0529 ± 0.0377
	Salicylate	3.29 ± 0.04	4.03 ± 0.35	−0.744 ± 0.042
	Hydrochlorothiazide[a]	–	–	–
	MTX	60.9 ± 2.7	3.97 ± 0.09	56.9 ± 2.7*
	Ofloxacin	1.17 ± 0.10	1.00 ± 0.31	0.180 ± 0.101
	Ciprofloxacin	1.11 ± 0.02	1.00 ± 0.19	0.110 ± 0.017
	Enoxacin	2.61 ± 0.27	2.60 ± 0.26	0.00587 ± 0.26727
3	Canrenoate	0.769 ± 0.009	1.06 ± 0.08	−0.293 ± 0.009
	Mefenamic acid	7.86 ± 0.72	8.35 ± 0.18	−0.491 ± 0.722
	Norfloxacin	2.05 ± 0.20	2.72 ± 0.20	−0.661 ± 0.196
	Piroxicam	3.99 ± 0.28	4.80 ± 0.07	−0.805 ± 0.279
	Meclofenamic acid	10.7 ± 0.4	13.4 ± 0.7	−2.79 ± 0.38
	MTX	67.1 ± 1.5	4.99 ± 0.16	62.1 ± 1.5*
	Theophylline	1.37 ± 0.07	1.15 ± 0.14	0.214 ± 0.068
	Caffeine	1.21 ± 0.07	1.13 ± 0.09	0.0791 ± 0.0674
	Nicorandil	0.521 ± 0.089	0.605 ± 0.131	−0.0833 ± 0.0893
4	Glutamic acid	1.32 ± 0.35	1.59 ± 0.37	−0.274 ± 0.347
	Warfarin	1.80 ± 0.10	2.13 ± 0.11	−0.328 ± 0.097
	Tranexamic acid	1.13 ± 0.09	1.50 ± 0.15	−0.368 ± 0.085
	Theobromine	0.732 ± 0.139	1.34 ± 0.38	−0.613 ± 0.139
	Neostigmine	4.02 ± 0.25	5.46 ± 0.34	−1.44 ± 0.25

Membrane vesicles (40 μg) prepared from MRP4-transfected or parental vector-transfected Sf9 cells (Mock) were incubated at 37°C for 60 min in uptake medium containing nine or ten compounds (each 5 μM) or 5 μM MTX only in the presence of 4 mM ATP. Each value represents the mean ± SEM (n=4).

*p<0.001, significantly greater than 0 μl/(mg protein·60 min) of MUI_D, Dunnett's test.

**p<0.001, significantly different from MUI_D of MTX (single), Dunnett's test.

[a] The authentic (10 pmol) sample was detected by LC–MS, while the compound was not detected in uptake samples of membrane vesicles prepared from MRP4-transfected and parental vector-transfected Sf9 cells.

osmotic pressure dependency was examined for the compounds identified by the first, second, and third screening studies.

As shown in Fig. 1(a, b and h), the uptake of CMZ, rebamipide and piperacillin by MRP4-expressing membrane vesicles in the presence of ATP (closed circle) was greater than that in the presence of AMP (open circle) under isotonic conditions. The uptake of CMZ and rebamipide decreased linearly with the increase in medium osmolarity. The y-intercept indicates the amount of compounds binding to the vesicle surface, MRP4 and/or filters. The y-intercept values for CMZ and rebamipide were less than 0 μl/(mg protein·min), indicating that the binding to the vesicle surface, MRP4 and/or filters is negligible. The uptake of piperacillin decreased linearly with the increase in medium osmolarity, and the y-intercept was 0.114 μl/(mg protein·min) (Table VI), indicating that 12% of piperacillin is bound to the vesicle surface, MRP4 and/or filters. Thus, these results indicate that CMZ, rebamipide and piperacillin were transported into the intravesicular space by MRP4 in an ATP-dependent manner.

Table V. MRP4-dependent single uptake of 17 compounds into membrane vesicles in the third screening

Compound	Vesicle/medium ratio (μl/(mg protein·30 min))		MUI_D μl/(mg protein·30 min)	Detection mode
	MRP4	Mock		
Furosemide	31.7 ± 2.6**	3.22 ± 0.14	28.5 ± 2.6	Negative
Ceftazidime	4.18 ± 0.09**	1.16 ± 0.10	3.02 ± 0.09	Positive
Pravastatin	3.19 ± 0.04**	0.856 ± 0.048	2.34 ± 0.04	Negative
Hydrochlorothiazide	2.97 ± 0.16**	1.52 ± 0.01	1.45 ± 0.16	Negative
Sulbactam	1.10 ± 0.14*	0.197 ± 0.014	0.901 ± 0.145	Negative
Baclofen	1.56 ± 0.05**	0.730 ± 0.035	0.827 ± 0.054	Positive
Bezafibrate	1.19 ± 0.06**	0.487 ± 0.017	0.700 ± 0.060	Positive
Alacepril	0.522 ± 0.014**	0.284 ± 0.017	0.237 ± 0.014	Positive
Dantrolene	3.30 ± 0.20	3.05 ± 0.07	0.247 ± 0.196	Negative
Amoxicillin	0.601 ± 0.013	0.438 ± 0.062	0.163 ± 0.013	Positive
Sulpyrine	2.97 ± 0.11	2.83 ± 0.06	0.137 ± 0.115	Positive
Ethacrynic acid	2.26 ± 0.02	2.16 ± 0.19	0.100 ± 0.018	Positive
Azathioprine	0.0959 ± 0.0077	0.0566 ± 0.0190	0.0393 ± 0.0077	Positive
Sulindac	0.301 ± 0.017	0.269 ± 0.028	0.0317 ± 0.0167	Positive
Captopril	0.162 ± 0.003	0.173 ± 0.002	−0.0116 ± 0.0027	Positive
Salicylate	1.02 ± 0.04	1.05 ± 0.05	−0.0363 ± 0.0395	Positive
Nalidixic acid	0.359 ± 0.073	0.415 ± 0.061	−0.0568 ± 0.0725	Positive

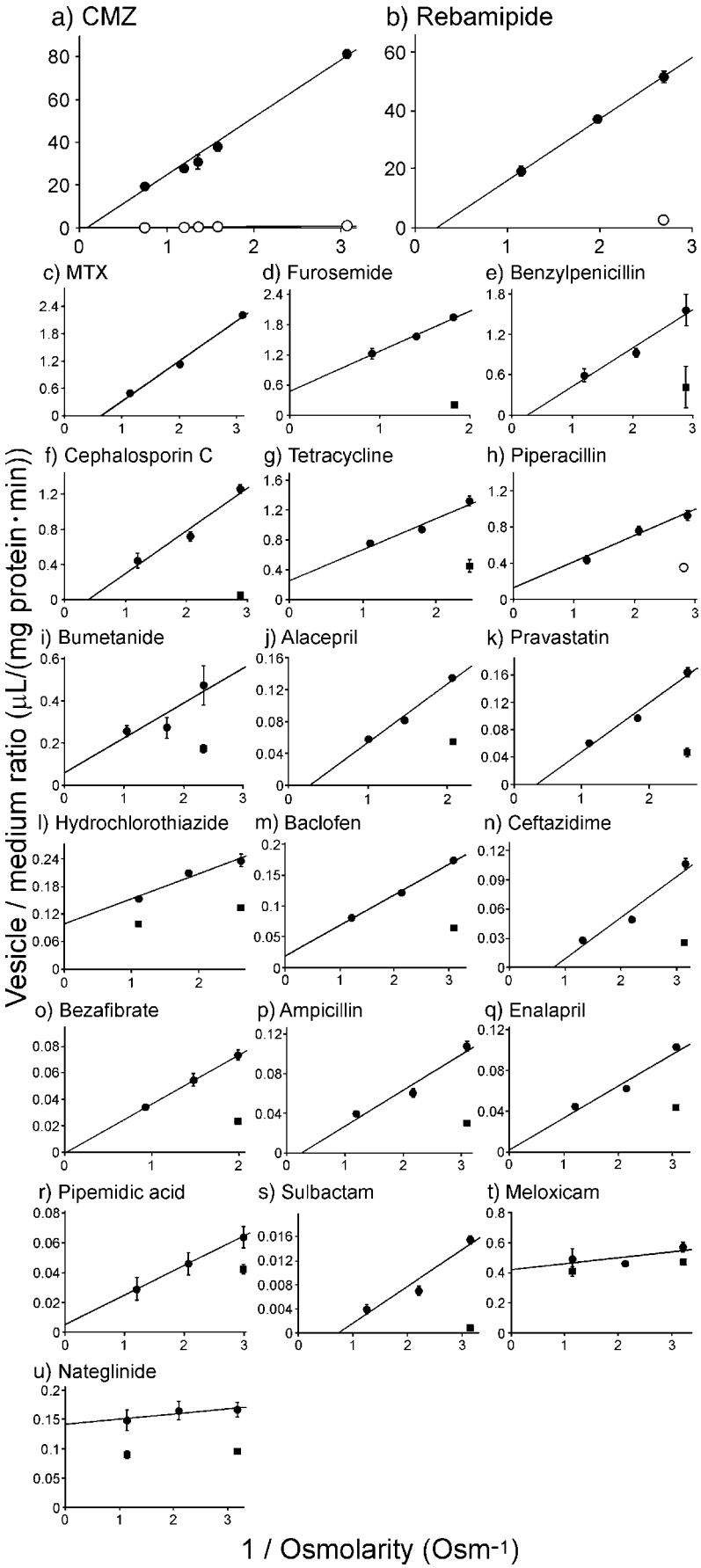
Membrane vesicles (10 μg) prepared from MRP4-transfected or parental vector-transfected Sf9 cells were incubated at 37°C for 30 min in uptake medium containing 10 μM single compound in the presence of 4 mM ATP. The uptakes of 12 compounds or five compounds were simultaneously determined by MRM multichannel LC–MS/MS in the ESI positive or negative mode, respectively. Each value represents the mean ± SEM (n=3).
*p<0.01, **p<0.001, significantly different from the vesicle-to-medium $ratio_{Mock}$, Student's t test.

We found that 17 compounds shown in Fig. 1(d–g, i–u) exhibited greater uptake by MRP4-expressing membrane vesicles (circles) than that by the control vesicles (squares) under isotonic conditions, and the uptake decreased linearly with the increase in medium osmolarity. Among these 17 compounds, the y-intercept values for eight compounds (Fig. 1; e, f, j, k, n, o, p and s) were less than 0 μl/(mg protein·min) (Table VI), indicating that these compounds (benzylpenicillin, cephalosporin C, alacepril, pravastatin, ceftazidime, bezafibrate, ampicillin and sulbactam) were transported into the intravesicular space by MRP4.

The y-intercept values for six other compounds, furosemide, tetracycline, bumetanide, baclofen, enalapril and pipemidic acid (Fig. 1; d, g, i, m, q and r) were 0.489, 0.267, 0.0529, 0.0191, 0.00303 and 0.00436 μl/(mg protein·min), respectively (Table VI), which indicate the binding amount of these compounds to the vesicle surface, MRP4 and/or filters. The apparent MRP4-dependent transport is the sum of the amount of compounds transported into the intravesicular space by MRP4 and that bound to MRP4, and is determined by subtracting the vesicle-to-medium $ratio_{Mock}$ from the vesicle-to-medium $ratio_{MRP4}$. As shown in Table VI, the y-intercept values of these six compounds were less than 31% of the apparent MRP4-dependent transport under isotonic conditions (1.74, 0.866, 0.299, 0.109, 0.0587 and 0.0218 μl/(mg protein·min) for furosemide, tetracycline, bumetanide, baclofen, enalapril and pipemidic acid, respectively). Therefore, the major part of the apparent MRP4-dependent transport does represent transport into the intravesicular space by MRP4, and so these six compounds are also substrates of MRP4.

The y-intercept values of the remaining three compounds, hydrochlorothiazide, meloxicam and nateglinide (0.0992, 0.426 and 0.141 μl/(mg protein · min)) were 97, 443 and 203% of the apparent MRP4-dependent transport under isotonic conditions (0.102, 0.0962 and 0.0694 μl/(mg protein · min)), respectively (Table VI). Thus, it appears that the apparent MRP4-dependent transport of these compounds can be mainly attributed to the binding to MRP4. To confirm this, the uptake by the control vesicles was examined under hypertonic conditions, and the apparent MRP4-dependent transport amounts were compared between isotonic and hypertonic conditions, since the binding to MRP4 is independent of osmotic pressure. As shown in Table VI, the apparent MRP4-dependent transport of hydrochlorothiazide under hypertonic conditions was significantly lower than that under isotonic conditions, indicating that hydrochlorothiazide is transported into the intravesicular space by MRP4. In contrast, for meloxicam and nateglinide, the apparent MRP4-dependent transport was similar under hypertonic and isotonic conditions, suggesting that meloxicam and nateglinide are not transported into the intravesicular space via MRP4, but rather bind to it.

Table VI summarizes the slope of the osmotic pressure dependency shown in Fig. 1 in order of the slope value. CMZ and rebamipide both showed elevated slopes of ATP-dependent transport into MRP4-expressing membrane vesicles (Table VI), confirming them to be strong substrates of human MRP4. The slopes of the osmotic pressure dependency for furosemide, benzylpenicillin, cephalosporin C, tetracycline, piperacillin, and bumetanide were of a similar order to that of MTX, which is a known substrate of MRP4. Alacepril, pravastatin, hydrochlorothiazide, baclofen, ceftazidime, bezafibrate, ampicillin, enalapril, pipemidic acid, and sulbactam were found to be relatively weak substrates of MRP4.


a) CMZ
b) Rebamipide
c) MTX
d) Furosemide
e) Benzylpenicillin
f) Cephalosporin C
g) Tetracycline
h) Piperacillin
i) Bumetanide
j) Alacepril
k) Pravastatin
l) Hydrochlorothiazide
m) Baclofen
n) Ceftazidime
o) Bezafibrate
p) Ampicillin
q) Enalapril
r) Pipemidic acid
s) Sulbactam
t) Meloxicam
u) Nateglinide
Vesicle / medium ratio (μL/(mg protein・min))
1 / Osmolarity (Osm-1)

◀ **Fig. 1.** Osmotic pressure dependency of the uptake of substrate candidates into MRP4-expressing membrane vesicles. The uptake of 1 μM of each substrate (CMZ (**a**), rebamipide (**b**), MTX (**c**), and piperacillin (**h**)) was measured at 37°C for 3 min by incubating membrane vesicles (10 μg) prepared from MRP4-transfected (circles) Sf9 cells in uptake medium containing 250 mM sucrose and various concentrations of raffinose (0–0.3 M, but 0–0.5 M for CMZ). The uptake of 5 μM of each substrate (benzylpenicillin (**e**), tetracycline (**g**), bumetanide (**i**), and ampicillin (**p**)), 10 μM substrate (furosemide (**d**), cephalosporin C (**f**), alacepril (**j**), pravastatin (**k**), hydrochlorothiazide (**l**), baclofen (**m**), bezafibrate (**o**), enalapril (**q**), pipemidic acid (**r**), sulbactam (**s**), meloxicam (**t**), and nateglinide (**u**)) or 100 μM ceftazidime (**n**) was measured at 37°C for 10 min by incubating membrane vesicles (10 μg) prepared from MRP4-transfected (*circles*) or parental vector-transfected (*squares*) Sf9 cells in uptake medium containing 250 mM sucrose and various concentration of raffinose (0–0.3 M). *Closed symbols*, uptake in the presence of 4 mM ATP; *open symbols*, uptake in the presence of 4 mM AMP. Each point represents the mean ± SEM (n=3–4)

Characterization of CMZ Transport by Human MRP4

As CMZ exhibited the highest transport activity among the compounds examined, its activity was further characterized. Fig. 2a shows the time-course of CMZ transport by MRP4-expressing membrane vesicles in the presence of ATP, demonstrating a linear phase for at least 3 min with an initial uptake rate of 60.6 μl/(mg protein·min). The vesicle/medium ratio of CMZ at 10 min by MRP4-expressing membrane vesicles in the presence of ATP (409 ± 9 μl/mg protein) was significantly greater than that in the presence of AMP (13.9 ± 0.5 μl/mg protein) or into the control vesicles in the presence of ATP (5.80 ± 0.04 μl/mg protein). A concentration-dependence was established for CMZ transport by MRP4-expressing membrane vesicles, and is illustrated in Fig. 2b. The K_m and V_{max} values (mean ± standard deviation (SD)) obtained for CMZ transport were 28.5 ± 3.0 μM and 0.914 ± 0.029 nmol/(mg protein·min), respectively. Table VII summarizes the inhibitory effects exerted by MTX and DHEAS, which were selected as known MRP4 substrates, and indomethacin, ketoprofen, and diclofenac, which were selected as known MRP4 inhibitors. All of the compounds had significant inhibitory effects on the transport of CMZ into human MRP4-expressing membrane vesicles (Table VII). MTX (30 μM) inhibited CMZ transport by 39.4%, suggesting that the IC_{50} value of MTX for MRP4-mediated CMZ transport is in the range of 30 μM to 100 μM. This range is similar to the reported IC_{50} value (100 to 200 μM) of MTX for MRP4-mediated estradiol 17 β-D-glucuronide transport (23).

DISCUSSION

The present study describes the establishment of a comprehensive and rapid substrate-screening method (LC–MS/MS cocktail method) for MRP4. By employing this sequential screening method, 18 new substrates for MRP4 were identified from a set of 49 compounds; furosemide and hydrochlorothiazide, which were recently reported to be substrates of MRP4 (24), were also confirmed to be substrates. Substrate screening for ABC transporters has previously been performed rapidly by means of ATPase assays and inhibition assays against the vesicular uptake of a known substrate. Azidothymidine, 9-(2-phosphonylmethoxyethyl)adenine, and MTX are transport substrates for MRP4, but they do not stimulate ATP hydrolysis in MRP4-expressing membrane preparations (17). Urate is a transport substrate of MRP4, but does not inhibit the transport of cAMP into MRP4-expressing membrane vesicles (25). Therefore, these assays may detect false positives as substrates. By contrast, a vesicle-uptake study evaluates the direct transport of the substrate via the transporter. Therefore, the LC–MS/MS cocktail method described here is more suitable for substrate identification than either the ATPase assay or the inhibition assay. Since vesicle-uptake studies have been performed for various ABC transporters, the LC–MS/MS cocktail method should be applicable for the identification of substrates of other ABC transporter subtypes.

However, standards (10 pmol) of four compounds were not detected (Table II), suggesting that the LC–MS/MS cocktail method may not be applicable to all the compounds tested. The molecular weights of these four compounds are less than 250 (Table I), and such compounds are often difficult to detect by LC–MS/MS because of high background noise. In addition, isosorbide dinitrate lacks functional groups producing positive ions, such as amino groups. Alendronate has an amino group, but also has double phosphate groups. Such physicochemical properties make these compounds difficult to ionize in the positive-ionization mode.

The LC–MS/MS cocktail method is characterized as follows. The first screening is used to comprehensively identify the potent and moderate substrates in a mixture of many compounds. The second screening is used to efficiently characterize non-substrates as well as substrates, since dividing the compounds into groups results in a reduction in the effect of mutual inhibition. The third screening classifies all the remaining compounds as substrates or non-substrates. However, even if MRP4-dependent transport appears to be present, if the effect of osmotic pressure on the uptake by membrane vesicles is not evaluated, a compound binding to MRP4 might be mistakenly identified as a substrate. Therefore, the fourth step is necessary to demonstrate that the candidate is a substrate. Indeed, meloxicam and nateglinide were apparently transported by MRP4 in the first screening procedure. However, they were finally identified as compounds that were merely bound to MRP4, since no sensitivity of the apparent MRP4-dependent transport to osmotic pressure was observed (Table VI). Higgins *et al.* proposed the ATP switch model for ABC transporters, in which the transport cycle of ABC transporters is initiated by substrate binding, leading to an increase of the affinity of the nucleotide binding domain for ATP (26). Based on this model, the binding of meloxicam and nateglinide is likely to be ATP-independent. However, ATP-dependent binding can not be ruled out, and further studies will be needed to elucidate in detail the binding mechanisms.

In the present study, 12 and eight substrate candidates were identified in the first and third screenings, respectively. In the primary first screening, potent substrates (such as CMZ and rebamipide) were mainly identified (Tables II and VI). In the secondary first screening, moderate substrates were mainly identified (Tables III and IV). Although eight

Table VI. Evaluation of osmolarity-dependent transport activities for the MRP4 substrate candidates into MRP4-expressing membrane vesicles

Compound	Screening step	Slope Osm·μl/(mg protein·min)	Intercept μl/(mg protein·min)	Apparent MRP4-dependent $transport_{isotonic}$ μl/(mg protein·min)	Apparent MRP4-dependent $transport_{hypertonic}$ μl/(mg protein·min)
CMZ	Primary first	2.76×10	<0	$8.06\times10 \pm 0.22\times10$[a]	
Rebamipide	Primary first	2.10×10	<0	$4.98\times10 \pm 0.20\times10$[a]	
MTX (reference)		8.76×10^{-1}	<0		
Furosemide	Third	7.93×10^{-1}	4.89×10^{-1}	1.74 ± 0.06	
Benzylpenicillin	Primary first	5.72×10^{-1}	<0	1.14 ± 0.23	
Cephalosporin C	Secondary first	4.81×10^{-1}	<0	1.22 ± 0.04	
Tetracycline	Primary first	4.15×10^{-1}	2.67×10^{-1}	$8.66\times10^{-1} \pm 0.66\times10^{-1}$	
Piperacillin	Primary first	2.86×10^{-1}	1.14×10^{-1}	$5.45\times10^{-1} \pm 0.57\times10^{-1}$[a]	
Bumetanide	Primary first	1.66×10^{-1}	5.29×10^{-2}	$2.99\times10^{-1} \pm 0.93\times10^{-1}$	
Alacepril	Third	7.35×10^{-2}	<0	$8.12\times10^{-2} \pm 0.05\times10^{-2}$	
Pravastatin	Third	7.13×10^{-2}	<0	$1.18\times10^{-1} \pm 0.07\times10^{-1}$	
Hydrochlorothiazide	Third	5.43×10^{-2}	9.92×10^{-2}	$1.02\times10^{-1} \pm 0.14\times10^{-1}$	$5.71\times10^{-2}\pm0.32\times10^{-2}$*
Baclofen	Third	5.00×10^{-2}	1.91×10^{-2}	$1.09\times10^{-1} \pm 0.04\times10^{-1}$	
Ceftazidime	Third	4.24×10^{-2}	<0	$8.00\times10^{-2} \pm 0.55\times10^{-2}$	
Bezafibrate	Third	3.71×10^{-2}	<0	$5.04\times10^{-2} \pm 0.38\times10^{-2}$	
Ampicillin	Primary first	3.62×10^{-2}	<0	$7.67\times10^{-2} \pm 0.50\times10^{-2}$	
Enalapril	Secondary first	3.11×10^{-2}	3.03×10^{-3}	$5.87\times10^{-2} \pm 0.24\times10^{-2}$	
Pipemidic acid	Secondary first	2.01×10^{-2}	4.36×10^{-3}	$2.18\times10^{-2} \pm 0.70\times10^{-2}$	
Sulbactam	Third	6.09×10^{-3}	<0	$1.47\times10^{-2} \pm 0.06\times10^{-2}$	
Meloxicam	Secondary first	3.72×10^{-2}	4.26×10^{-1}	$9.62\times10^{-2} \pm 2.91\times10^{-2}$	$8.14\times10^{-2}\pm6.79\times10^{-2}$
Nateglinide	Secondary first	8.75×10^{-3}	1.41×10^{-1}	$6.94\times10^{-2} \pm 1.28\times10^{-2}$	$5.85\times10^{-2}\pm1.80\times10^{-2}$

The apparent MRP4-dependent $transport_{isotonic}$ was obtained by subtracting the vesicle-to-medium $ratio_{mock}$ from the vesicle-to-medium $ratio_{MRP4}$ in the absence of raffinose. The apparent MRP4-dependent $transport_{hypertonic}$ was obtained in the presence of 0.3 M raffinose. Each value was determined from the same data shown in Fig. 1. Each value of the apparent MRP4-dependent transport represents the mean ± SEM.

*$p<0.05$, significantly different from the apparent MRP4-dependent transport $_{isotonic}$, Student's t test.

[a] The apparent MRP4-dependent transport $_{isotonic}$ was obtained by subtracting the vesicle-to-medium ratio $_{MRP4}$ in the presence of AMP from that in the presence of ATP, in the absence of raffinose.

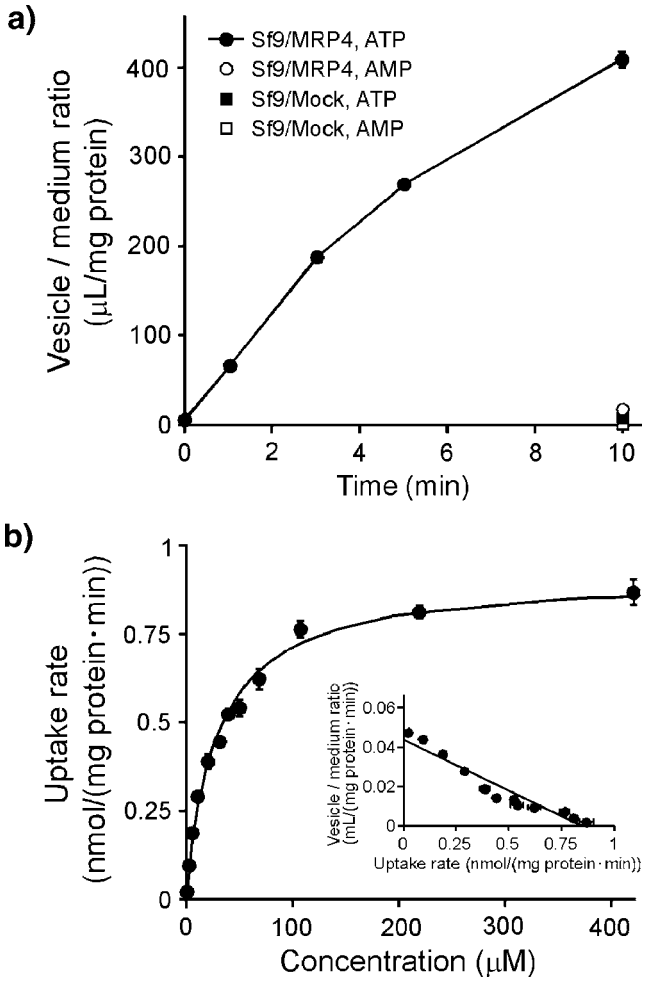


Fig. 2. Time-dependent and concentration-dependent transport of CMZ by MRP4. Membrane vesicles (10 μg) prepared from MRP4-transfected or parental vector-transfected Sf9 cells (Mock) were incubated at 37°C in uptake medium containing CMZ in the presence of 4 mM ATP or 4 mM AMP. **a**, time dependence of 1 μM CMZ uptake by MRP4. **b**, concentration-dependence of CMZ uptake by MRP4. The uptake rates were measured at 3 min, and were calculated by subtracting the values obtained in the presence of 4 mM AMP from those obtained in the presence of 4 mM ATP. *Inset*: Eadie–Hofstee plot of the same data. Each point represents the mean ± SEM (n=3–4)

substrates were identified in the third screening, most had a weak ability to be transported by MRP4 (Tables V and VI). These results demonstrated that the first screening was useful for comprehensively identifying potent substrates. In contrast, the entire process (including the third screening) was necessary to identify all the substrates, since not all of substrates were identified in the first screening.

For the second screening, the compounds were divided into four groups. If the compounds were divided randomly, substrates, inhibitors and non-substrates would be equally distributed in all groups, and it is expected that none of the compounds is characterized as substrate candidates or non-substrates due to mutual inhibition in all groups. As a result, all compounds would have to be tested individually in the third screening. To minimize this problem, compounds that were expected to inhibit MRP4 were classified into group 2 or 3, so that the compounds of groups 1 and 4 could be efficiently characterized as substrate candidates or non-substrates.

β-Lactam antibiotics (amoxicillin and ceftazidime), ACE inhibitors (alacepril and captopril) and furosemide may inhibit MRP4 transport, because their structural analogues were identified as substrate candidates in the first screening. NSAIDs (sulindac, salicylate, mefenamic acid, piroxicam and meclofenamic acid), quinolone antibiotics (ofloxacin, ciprofloxacin, enoxacin and norfloxacin) and pravastatin may also inhibit MRP4 transport, since their structural analogues have been reported to be inhibitors of MRP4 and/or to interact with MRP2, which is a major subtype of MRPs (27–29). These compounds were classified into group 2 or 3. As a result, 16 compounds in groups 3 and 4 were identified to be non-substrates, since no significant MRP4-dependent transport was observed for any of them under the condition of no inhibition of MTX transport via MRP4 (Table IV). Although the actual inhibitory effect in each group was contrary to our expectation, the second screening efficiently identified the non-substrates and decreased the number of compounds for the third screening. There still remains a possibility that these groups included compounds that did not inhibit MTX transport, but did inhibit the transport of other substrates via MRP4. Thus, the possibility of missing substrates cannot be completely ruled out.

The first and second screenings were performed by Q1 multichannel LC–MS, which did not require optimization of

Table VII. Inhibitory effects of known substrates and inhibitors for MRP4 on CMZ uptake into MRP4-expressing membrane vesicles

Inhibitor	Concentration μM	Vesicle/medium ratio μl/(mg protein·3 min)	% of control
Control		208 ± 4	100 ± 2
Indomethacin	50	11.9 ± 0.3	5.72 ± 0.14*
Ketoprofen	50	66.2 ± 2.9	31.7 ± 1.4*
MTX	30	127 ± 5	60.6 ± 2.4*
DHEAS	3	143 ± 4	68.7 ± 2.0*
Diclofenac	100	166 ± 8	79.3 ± 3.9*

Membrane vesicles (10 μg) prepared from MRP4-transfected Sf9 cells were incubated at 37°C for 3 min in uptake medium containing 1 μM CMZ and 4 mM ATP in the presence or absence of the indicated inhibitors. The CMZ uptake into membrane vesicles prepared from parental vector-transfected Sf9 cells in the presence of 4 mM ATP was subtracted from all uptake values. Each value represents the mean ± SEM (n=4).
*p<0.001, significantly different from the control uptake, Dunnett's test.

the measurement conditions for each compound. A simultaneous measurement was achieved simply by setting a common ionization condition and *m/z* channels assumed to be [monoisotopic mass + 1]. Thus, Q1 multichannel LC–MS is a simple and rapid quantification method, and it is suitable for substrate screening requiring speed rather than accuracy. In contrast, MRM multichannel LC–MS/MS, which was used in the third and fourth screenings, requires optimization of the MRM channel of each analyte before the simultaneous measurement. Despite requiring time for optimization, it has an approximately 100-fold higher sensitivity than Q1 multichannel LC–MS, allowing the use of small amounts of membrane vesicles. Therefore, MRM multichannel LC–MS/MS is suitable for the precise identification of substrates by individual transport studies for many compounds.

This study showed that CMZ and rebamipide are potent substrates of MRP4 compared with MTX and 16 further substrates. The reported MRP4 substrates (with K_m values <100 μM) were mainly endogenous compounds, such as steroids, prostaglandins, and cyclic nucleotides, and included only one drug, topotecan (K_m=1.66 μM) (1,30). Transport of CMZ by MRP4 exhibited a K_m value of 28.5 μM, indicating that CMZ has a relatively high affinity for MRP4 among drugs. The intravesicular volume of membrane vesicles prepared from various species was reported to be from 0.60 to 10 μl/mg protein (31–33), although that of Sf9 cell membrane vesicles is not known. These results suggest that the uptake of CMZ and rebamipide into membrane vesicles is an uphill process against a concentration gradient, since the vesicle-to-medium ratios of these compounds are much greater than the reported intravesicular volume, as shown in Figs. 1b and 2a (rebamipide, 155 μl/mg protein at 3 min; CMZ, 409 μl/mg protein at 10 min). This supports the conclusion that these drugs are potent substrates of MRP4.

Based on the creatinine clearance (approximately 100 ml/min) and the binding ratio to serum protein (84% for CMZ and 98% for rebamipide) (34,35), the clearance rates of CMZ and rebamipide attributable to glomerular filtration alone can be calculated to be 16 and 2 ml/min respectively. These values are far smaller than the apparent renal clearances of CMZ and rebamipide, which have been reported to be 111 and 287 ml/min, respectively (36,37). This implies that CMZ and rebamipide are mainly eliminated by tubular secretion, and that high expression of MRP4 at the apical membrane of the proximal tubules plays an important role in the elimination process.

A recent study showed that the mRNA level of MRP4 in the kidney is the highest among the transporters (including MRP2, MRP4, OAT4, and ABCG2) that contribute to the apical efflux transport of organic anions in the proximal tubules (4). However, the identified drug substrates for MRP4 have been limited to a few types of compound, such as nucleotide, camptothecin, and folate analogs (1). The present study has identified 18 drugs as substrates for MRP4, and revealed that MRP4 transports various anionic drugs, including β-lactam antibiotics, diuretics, and ACE inhibitors. It was previously reported that these drugs are eliminated by tubular secretion from the kidney (6,7,9), and Mrp4 is involved in the apical efflux of anionic drugs, such as adefovir and tenofovir, *in vivo* (3). The present study suggests that MRP4 is, at least partially, involved in the apical efflux of various anionic drugs, including β-lactam antibiotics, diuretics, and ACE inhibitors.

The concentration of the centrally acting antispastic agent, baclofen, in the brain interstitial fluid (ISF) was 27-fold lower than that in the plasma, and the restricted distribution in the brain ISF is ascribed to the efficient efflux from the brain through the blood–brain barrier (BBB) (11). We suggested that OAT3, localized at the abluminal membrane of the brain capillary endothelium, promotes the entry of baclofen into the endothelium from the brain (38). Our present study suggested that MRP4, localized at the luminal membrane (39), is involved in the efflux transport of baclofen into the plasma from the endothelium. Although P-glycoprotein (P-gp) plays an important role in the efflux transport of various drugs at the luminal membrane, it has not been reported to transport baclofen. Additionally, it has been reported that the concentration of baclofen in the brain ISF was markedly increased by administration of probenecid, which is an inhibitor not of P-gp, but of MRP (11,40). Consequently, MRP4 might be one of the important efflux routes of baclofen at the BBB.

The distribution of β-lactam antibiotics in the brain is restricted, resulting in reduced neurotoxicity and difficulties in treating bacterial CNS infections (13,41). Recent studies have suggested that both OAT3, which is localized at the abluminal membrane of the BBB and the apical membrane of the blood–cerebrospinal fluid barrier (BCSFB), and peptide-transporter 2 (PEPT2), which is localized at the apical membrane of the BCSFB, contribute to the restricted distribution of β-lactam antibiotics in the brain (42–44). However, no efflux transporter of β-lactam antibiotics has been identified on the blood side. In the present study, we identified β-lactam antibiotics as substrates of MRP4, which is an efflux transporter localized on the blood side of the BBB and BCSFB. Therefore, our present findings will improve our understanding of the restricted distribution of β-lactam antibiotics in the brain.

In conclusion, the present study demonstrated that the LC–MS/MS cocktail method is useful for comprehensive substrate screening for MRP4, and identified 18 compounds as new substrates of human MRP4. Furthermore, it identified CMZ and rebamipide as potent substrates. Using this methodology, we established a new substrate profile for MRP4. Our results indicate that MRP4 is a new efflux route for various anionic drugs in the kidney, the BBB and the BCSFB. Our approach is therefore expected to be widely useful for the characterization of ABC transporter function. Furthermore, it should also be useful for lead optimization from compound banks during early drug development.

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