Screening to Identify Multidrug Resistance-Associated Protein Inhibitors with Neuroblastoma-Selective Cytotoxicity

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The aim of the present study is to discover multidrug resistance-associated protein (MRP) inhibitors with neuroblastoma-selective cytotoxicity by means of fluorescence assay with a membrane-permeable fluorescent dye, Fluo-8 AM, based on our observation that gene expression of Mrp3 in neuroblastoma Neuro2a cells was remarkably higher than that in primary cultured cortical neurons, as determined by real-time PCR. Neuro2a cells showed minimal fluorescence upon incubation with Fluo-8 AM. However, blocking of Mrp3 efflux function by small interfering RNA (siRNA) transfection or inhibition with probenecid resulted in significant dye accumulation, observed as an increase of fluorescence. Interestingly, Mrp3 siRNA or probenecid treatment also resulted in increased cytotoxicity, as evidenced by decreased 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-reducing activity of Neuro2a, with a concomitant increase in release of lactate dehydrogenase. On the other hand, primary cultured neurons exhibited higher fluorescence intensity after incubation with Fluo-8 AM regardless of addition of probenecid. Also, probenecid only minimally affected MTT-reducing activity. Thus, probenecid showed selective cytotoxicity towards Neuro2a cells. Based on these findings, we screened a series of established therapeutic agents for ability to induce Fluo-8 accumulation in Neuro2a cells. Several uricosuric and nonsteroidal anti-inflammatory drugs were identified, and these drugs were confirmed to decrease MTT-reducing activity selectively in Neuro2a. There was a negative linear correlation between Fluo-8 accumulation and cytotoxicity of these agents. Although the compounds identified here are insufficiently potent for practical application, further screening to discover higher-affinity MRP3 inhibitors using larger chemical libraries may uncover drug candidates with potent neuroblastoma-selective cytotoxicity.

Key words neuroblastoma; neuron; multidrug resistance-associated protein; cytotoxicity; fluorescence

Neuroblastoma is one of the most common extracranial tumors in childhood,1–3 together with leukemia, brain cancer and malignant lymphoma.2,4 The prognosis is mostly good, even at the advanced stage, if neuroblastoma is found before one year of age, whereas the prognosis is generally worse in older children.3–5 Surgery, radiation therapy, chemotherapy and/or immunotherapy are used in the treatment of high-risk neuroblastoma,6,7 but improvement of the cure rates is still desirable. In addition, chemotherapy for neuroblastoma can cause neurotoxicity,7 which may require discontinuation of treatment.8,9 Therefore, it is desirable to develop therapeutic agents that exhibit neuroblastoma-selective cytotoxicity via a mechanism different from those of existing drugs, with minimal neurotoxicity.

Some transporters are highly expressed in cancer cells compared to normal cells, and inhibitors of these transporters have recently been highlighted as possible anticancer drugs. For example, L-type amino acid transporter (LAT) 1 supplies essential amino acids that cannot be synthesized by intracellular metabolism in cancer cells.10 LAT1 is highly expressed in various cancer cells, including hepatocellular carcinoma, breast cancer and prostatic carcinoma, whereas its expression is minimal in most normal cells.11 Thus, LAT1-selective inhibitors have been developed as anticancer drugs with minimal cytotoxicity to normal cells.12 Glucose transporter (GLUT) is also highly expressed in cancer cells compared to normal cells,13 and could be another target for cancer chemotherapy; indeed, anticancer activity of GLUT inhibitors has been reported.14,15 Oligopeptide transporter (PEPT) 2 is highly expressed in chronic myelogenous leukemia stem cells, and pharmacological inhibition of this transporter resulted in a reduction of cellular activity.16 Transporters generally regulate intracellular-to-extracellular concentration ratios of various nutrients and/or metabolites, and some of them play fundamental roles in cell homeostasis. Thus, they could be promising targets for cancer treatment, and their selective expression in cancer cells may mean that undesired cytotoxicity to normal cells would be minimal.

Transporters are categorized into two superfamilies, ATP-binding cassette (ABC) and solute carrier (SLC) transporters, which include LAT, GLUT and PEPT. ABC transporters actively exclude substrates from the intracellular space, whereas SLC transporters are generally involved in both influx and efflux transport of their substrates. Inhibition of some SLC transporters has been reported to have anticancer effects.12,14,15,17 On the other hand, most work on ABC transporters has been focused on ABC transporters that mainly recognize xenobiotics and mediate elimination of anticancer drugs used in chemotherapy. We were interested in the possibility that inhibitors of ABC xenobiotic transporters might show selective cytotoxicity towards neuroblastoma, with minimal neurotoxicity.

In the present study, we used the mouse neuroblastoma-derived cell line Neuro2a cells and mouse primary cultured cortical neurons as models of neuroblastoma cells and neurons, respectively. Neuro2a are one of the most widely used neuroblastoma models.18,19 On the other hand, primary cultured cortical neurons have been used to compare cytotoxic-
poly-L-lysine and cytosine arabinoside were purchased culture medium by using a 1000 µL pipette tip and plated at a density of 1.5×10^5 cells/cm² on plastic dishes coated with 7.5 µg/mL poly-L-lysine. Cortical neurons were cultivated in DMEM supplemented with 5% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, 28 mM glucose, 2 mM glutamine, 5 mM N-(2-hydroxyethyl)-piperazine-N′-2-ethanesulfonic acid (HEPES), 25 µg/mL apo-transferrin, 250 ng/mL insulin, 0.5 µM β-estradiol, 1.5 mM triiodothyronine, 10 nM progesterone, 4 ng/mL sodium selenite and 50 µM putrescine for the initial 4 h at 37°C in a humidified 5% CO₂ incubator. Further culture was performed in DMEM supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, 28 mM glucose, 2 mM glutamine, 5 mM HEPES, 50 µg/mL apo-transferrin, 500 ng/mL insulin, 1 µM β-estradiol, 3 mM triiodothyronine, 20 nM progesterone, 8 ng/mL sodium selenite and 100 µM putrescine up to 3 d in vitro (DIV). During 2 to 3 DIV, cells were treated with 25 µM cytosine arabinoside for 12 h to reduce proliferating cells. Under these culture conditions, more than 99% of cells were immunoreactive with antibody against microtubule-associated protein 2. 21)

Mouse neuroblastoma-derived cell line Neuro2a cells were plated on at 1.0×10^5 cells/mL and cultured in DMEM supplemented with 10% FBS for 3 DIV. Cultures were maintained in a humidified atmosphere of 5% CO₂-95% air at 37°C. When knockdown of Mrp3 was performed, Neuro2a cells were transiently transfected with siRNA or negative control siRNA by using lipofectamine RNAiMAX in Opti-MEM according to the manufacturer’s instructions. Culture medium was replaced with DMEM supplemented with 10% FBS at 24 h after the transfection, and culture was continued for an additional 2 DIV.

**Quantitative Reverse Transcription (RT)-PCR** Total RNA was extracted from cultured cells according to the standard ISOGEN procedure. cDNA was synthesized with oligo(dT)₂₅ primer, deoxynucleotide triphosphate mix, RT buffer and MultiScribe™ Reverse Transcriptase, and amplified on a Mx3005P (Agilent Technologies, Santa Clara, CA, U.S.A.) in a reaction mixture containing cDNA with relevant sense and antisense primers (Table 1), and THUNDERBIRD SYBR qPCR Mix. PCR reactions were initiated by template denaturation at 95°C for 15 min, followed by 40 cycles of amplification (denaturation at 95°C for 10 s, and primer annealing and extension at 60°C for 30 s). Relative quantification of expression levels of the target genes was determined by the delta-delta Ct method using transcripts of acidic ribosomal phosphoprotein P0 (36B4) as the internal standard.

**Fluorescence Assay for Mrp3 Transport Activity** Fluorescence assay using Fluo-8, a Ca²⁺-sensitive fluorescent dye.

### MATERIALS AND METHODS

**Materials** Dulbecco’s modified Eagle’s medium (DMEM), poly-L-lysine and cytosine arabinoside were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Fetal bovine serum (FBS) was obtained from Biowest (Nuaillé, France). isoGEN was provided by Nippon Gene (Tokyo, Japan). THUNDERBIRD SYBR qPCR Mix was supplied by TOYOBO (Osaka, Japan). All other chemicals and reagents were of the highest purity available and were purchased from commercial sources.

**Animals** Pregnant ICR mice were purchased from Sankyo Labo Service Co. (Toyama, Japan). The mice were housed under pathogen-free conditions at controlled temperature (21–25°C) with a 12 h light/dark cycle. The lights remained on from 8:00 to 20:00, and food and water were available ad libitum. The animals were cared for in strict compliance with the guidelines outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All animal procedures were approved by the Kanazawa University Animal Care Committee.

**Cell Cultures** Primary cortical neuronal cultures were prepared according to the method of Nakamichi et al. In brief, cerebral cortices from 15-d-old embryonic ICR mice were dissected and incubated with 0.25% trypsin in 210 µL pipette tip and plated at a density of 1.5×10^5 cells/cm² on plastic dishes coated with 7.5 µg/mL poly-L-lysine. Cortical neurons were cultivated in DMEM supplemented with 5% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, 28 mM glucose, 2 mM glutamine, 5 mM N-(2-hydroxyethyl)-piperazine-N′-2-ethanesulfonic acid (HEPES), 25 µg/mL apo-transferrin, 250 ng/mL insulin, 0.5 µM β-estradiol, 1.5 mM triiodothyronine, 10 nM progesterone, 4 ng/mL sodium selenite and 50 µM putrescine for the initial 4 h at 37°C in a humidified 5% CO₂ incubator. Further culture was performed in DMEM supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, 28 mM glucose, 2 mM glutamine, 5 mM HEPES, 50 µg/mL apo-transferrin, 500 ng/mL insulin, 1 µM β-estradiol, 3 mM triiodothyronine, 20 nM progesterone, 8 ng/mL sodium selenite and 100 µM putrescine up to 3 d in vitro (DIV). During 2 to 3 DIV, cells were treated with 25 µM cytosine arabinoside for 12 h to reduce proliferating cells. Under these culture conditions, more than 99% of cells were immunoreactive with antibody against microtubule-associated protein 2. Mouse neuroblastoma-derived cell line Neuro2a cells were plated on at 1.0×10^5 cells/mL and cultured in DMEM supplemented with 10% FBS for 3 DIV. Cultures were maintained in a humidified atmosphere of 5% CO₂-95% air at 37°C. When knockdown of Mrp3 was performed, Neuro2a cells were transiently transfected with siRNA or negative control siRNA by using lipofectamine RNAiMAX in Opti-MEM according to the manufacturer’s instructions. Culture medium was replaced with DMEM supplemented with 10% FBS at 24 h after the transfection, and culture was continued for an additional 2 DIV.

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**Fluorescence Assay for Mrp3 Transport Activity** Fluorescence assay using Fluo-8, a Ca²⁺-sensitive fluorescent dye.
was performed to measure Mrp3 transport activity according to the method of Nakamichi et al. with minor modifications. Cultured cells were washed with recording medium containing 129 mM NaCl, 4 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 4.2 mM glucose and 10 mM HEPES (pH 7.4) once and incubated at 37°C for 1 h in recording medium containing 0.02% Pluronic F-127 and 2 μM Fluo-8 AM, which is a membrane-permeable form of Fluo-8, in either the presence or absence of MRP inhibitors. Cultures were then washed with recording medium twice, followed by settlement for 20 min in the recording medium before observation with a confocal laser scanning microscope or measurement with a fluorescence microplate reader. The calcium ionophore A23187 at 20 μM was added 5 min before the observation or measurement in order to obtain maximal fluorescence intensity. Dye-loaded cells were monitored for fluorescence visualization with a confocal laser-scanning microscope (LSM710; Carl Zeiss, Jena, Germany) equipped with the same settings were maintained throughout the experiments. Fluorescence images were quantified as the mean of the whole screen using ImageJ software. The fluorescence intensity was also measured with a fluorescence microplate reader (MTP-880Lab; Corana Electric, Hitachinaka, Japan) at excitation and emission wavelengths of 490 and 530 nm, respectively.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Assay Mitochondrial activity as an index of cell survival was determined by using the MTT assay according to the method of Nakamichi et al. with minor modifications. In brief, cultured cells were washed once with PBS and incubated with MTT solution (0.5 mg/mL in PBS) for 1 h at 37°C. Subsequently, solubilizing solution (0.04 M HCl in isopropanol) at equivalent volume to the MTT solution was added, and the mixture was well shaken for 10 min to dissolve the formazan. The absorbance of the dissolved suspension at 570 nm was measured with a microplate reader.

Lactate Dehydrogenase (LDH) Assay LDH released into the medium, used as an index of cytotoxicity, was determined by LDH assay according to the method of Abe et al. with minor modifications. In brief, the culture medium was centrifuged at 16,400×g for 5 min at 4°C. After the centrifugation, 50 μL aliquots of the supernatant were collected in a 96-well plate and incubated with an equal volume of reaction mixture (2.5 mg/mL lactate, 2.5 mg/mL β-nicotinamide adenine dinucleotide, 600 μM MTT and 100 μM 1-methoxyphenazinc methosulfate diluted with 200 mM Tris–HCl, pH 8.2) for 15 min at 37°C. The reaction was stopped by adding 100 μL of stop buffer (50% N,N-dimethylformamide (DMF)/20% sodium docetyl sulfate (SDS), pH 4.7). The absorbance was measured with a microplate reader at a test wavelength of 570 nm and a reference wavelength of 655 nm.

Statistical Analysis All experiments were performed at least three times. Data are expressed as the mean±standard error of the mean (S.E.M.). The statistical significance of differences was determined by means of Student’s t-test. Correlation was assessed with nonparametric Spearman’s rank correlation test. p<0.05 was regarded as denoting a significant difference.

**RESULTS**

Expression Profiles of ABC Transporters in Neuroblastoma Cells and Neurons In order to identify ABC xenobiotic transporter(s) expressed at higher levels in neuroblastoma cells compared to neurons, we first examined the mRNA expression levels of various ABC xenobiotic transporters in mouse primary cultured cortical neurons and mouse neuroblastoma-derived cell line Neuro2a cells. Expression levels of mrp1, mrp3, mdr1a and mdr1b in Neuro2a cells were significantly higher than those in primary cultured neurons (Fig. 1). In particular, expression levels of mrp3 and mdr1b in Neuro2a cells were about 70 and 50 times higher than in primary cultured neurons, respectively (Fig. 1). The expression levels of mrp4, mrp5 and bcrp in Neuro2a cells were significantly lower than those in primary cultured neurons, while expression levels of mrp2 and mrp6 were under the quantitative detection limit (<60 copies/μg total RNA) in both cells (Fig. 1). Thus, we focused on mrp3 as a target ABC xenobiotic transporter for obtaining neuroblastoma-selective toxicity.

**Knockdown of Mrp3 in Neuro2a Cells** To examine whether inhibition of Mrp3 induces cellular toxicity in neuroblastoma, we performed knockdown of Mrp3 with siRNA in Neuro2a cells. The mRNA expression of mrp3 was markedly reduced at 48 h after transfection of siMrp3 in Neuro2a cells, compared to non-specific siRNA-transfected cells (Fig. 2A). To confirm that the knockdown of Mrp3 inhibits its transport function, we next assessed accumulation of fluorescent dye Fluo-8. The number of cells with marked fluorescence in the siMRP3-treated group was larger than that in the negative control group (Fig. 2B). Quantification of the fluorescence intensity of the cells with Image software showed that fluorescence in the siMrp3-treated group was much higher than that in the negative control group (Fig. 2C). Then, to establish whether knockdown of Mrp3 induces cytotoxicity in Neuro2a cells, we performed MTT and LDH assays of siMrp3-transfected cells. Knockdown of Mrp3 significantly decreased MTT-reducing activity of Neuro2a cells (Fig. 2D), with a concomitant increase of LDH release into the culture medium (Fig. 2E).
Thus, inhibition of Mrp3 suppresses efflux of Fluo-8 and induces cytotoxicity in Neuro2a cells.

Selective Toxicity of Mrp3 Inhibition in Neuro2a Cells with Minimal Neurotoxicity

We next compared the functional expression of Mrp3 between neuroblastoma and neurons in order to obtain insight into the selectivity of cytotoxicity provoked by Mrp3 knockdown. Fluorescence intensity of Fluo-8 after preincubation of Neuro2a cells and primary cultured neurons with Fluo-8 AM was measured in the presence or absence of the Mrp3 inhibitor probenecid. In Neuro2a cells, fluorescence intensity was minimal in the absence of probenecid (Fig. 3A), but exposure to probenecid markedly increased the number of cells showing high fluorescence (Fig. 3B). On the other hand, many cells with high fluorescence intensity were observed in primary cultured neurons, regardless of the addition of probenecid (Figs. 3C, D). Quantitative analysis was performed for these data, and the result was shown in Fig. 3E, revealing that fluorescence intensity in Neuro2a cells was remarkably increased by the addition of probenecid whereas fluorescence intensity in cultured neurons was scarcely affected by probenecid (Fig. 3E). These results suggest that functional activity of Mrp3 is much greater in Neuro2a cells than in primary cultured neurons.

Next, we attempted to establish whether inhibition of Mrp3 function using Mrp inhibitor drugs results in selective cytotoxicity to neuroblastoma cells. MTT-reducing activity was examined after exposure of Neuro2a cells and primary cultured neurons to various concentrations of Mrp inhibitor, probenecid or sulfinpyrazone, for 8 h in the absence of Fluo-8 (Figs. 4A, B). The addition of probenecid (Fig. 4A) or sulfinpyrazone (Fig. 4B) at a concentration of 1000 µM or more decreased the MTT-reducing activity in Neuro2a cells, but had a minimal effect in primary cultured neurons, suggesting that Mrp inhibitors are more cytotoxic to neuroblastoma cells than to neurons. On the other hand, the addition of probenecid (Fig. 4C) or sulfinpyrazone (Fig. 4D) in the presence of Fluo-8 for 1 h increased the fluorescence intensity of Fluo-8 in Neuro2a cells in a dose-dependent manner, and the fluorescence intensity at concentrations inducing selective cytotoxicity (ca. 1000 µM) was about 10–20 times higher than that in the absence of the inhibitor (Figs. 4C, D). These results suggest that inhibition of Mrp3 may be selectively cytotoxic to neuroblastoma cells, with minimal neurotoxicity, and the fluorescence intensity of Fluo-8 accumulated in Neuro2a cells may be a
surrogate parameter of the cytotoxicity to neuroblastoma.

**Fluorescence Screening of Mrp3-Inhibitory Activity to Find Drugs Showing Selective Cytotoxicity in Neuro2a Cells**

In order to find drugs with selective toxicity towards Neuro2a cells among clinically used drugs, we performed Fluo-8 accumulation assay in the presence of 75 candidate agents at various concentrations. All the candidate agents except MK571 are clinically used therapeutic agents, and include organic acids that would potentially be recognized by Mrps (Table 2). The fluorescence intensity of Fluo-8 in the presence of each drug was evaluated as a percentage of that in the presence of 2.5 mM probenecid (Table 2). Diclofenac sodium, flurbiprofen, mefenamic acid, sulfipyrazone and etodolac at 1 mM, and benzbromarone at 50 µM increased the fluorescence intensity to a level comparable to that in the presence of 2.5 mM probenecid (Table 2). In addition to these drugs, many uricosuric drugs and NSAIDs increased the fluorescence intensity to over 50% of that in the presence of 2.5 mM probenecid (Table 2).

We next focused on the uricosuric drugs and NSAIDs. Neuro2a cells were exposed to them at various concentrations for 8 h in the absence of Fluo-8, and the MTT-reducing activity was measured. The MTT-reducing activity was negatively correlated with fluorescence intensity observed in Fluo-8 accumulation assay, with a correlation coefficient of −0.544 (p<0.05; Fig. 5), suggesting an association between Mrp inhibition by these agents and the cytotoxic effects in Neuro2a cells. In particular, 2.5 mM probenecid, 1 mM sulfipyrazone,
sulindac, etodolac, phenylbutazone, flurbiprofen, mefenamic acid and dicrofenac sodium, and 50 µM benzbromarone greatly increased fluorescence intensity and decreased MTT-reducing activity (Fig. 5). Thus, these drugs are candidate agents to exhibit neuroblastoma-selective cytotoxicity.

**Selective Cytotoxicity of Clinically Used Drugs to Neuroblastoma**

To establish whether the candidate drugs exhibit neuroblastoma-selective cytotoxicity, primary cultured neurons were also exposed to various concentrations of the NSAIDs selected in the screening assay for 8 h, and the MTT-reducing activity was compared to that in Neuro2a cells (Fig. 6). Exposure to 1000 µM sulindac (Fig. 6A), 100 µM mefenamic acid (Fig. 6B), 100 and 1000 µM flurbiprofen (Fig. 6C) and 1000 µM phenylbutazone (Fig. 6E) significantly decreased the MTT-reducing activity of Neuro2a cells without causing a significant decrease in that of primary cultured neurons. MTT-reducing activity after exposure of Neuro2 cells to 1000 µM mefenamic acid (Fig. 6B) was lower than that of cultured neurons, but this drug and etodolac at 1000 and 2500 µM decreased the MTT-reducing activity of not only Neuro2a, but also cultured neurons (Figs. 6B, D). The MTT-reducing activity of primary cultured neurons was significantly increased at some concentrations of sulindac and etodolac (Figs. 6A, D).

**DISCUSSION**

We found here that siRNA knockdown of Mrp3 resulted in selective cytotoxicity of neuroblastoma Neuro2a cells, compared to primary cultured cortical neurons (Fig. 2). Further, inhibition of Mrp3 function by established therapeutic agents, such as probenecid, sulfipyrazone, sulindac, mefenamic acid, flurbiprofen and phenylbutazone, at appropriate concentrations resulted in selective cytotoxicity to Neuro2a cells with minimal toxicity to primary cultured neurons (Figs. 4, 6). Our results indicate that this ABC transporter may be a promising target molecule for neuroblastoma treatment. Indeed, MRP1, 3 and 4 are highly expressed in rodent and human neuroblastoma and are involved in efflux of anticancer drugs. High expression of MRP1 or MRP4 was reported to be associated with a poor prognosis of neuroblastoma. Henderson et al. also proposed that MRPs may be involved in exacerbation of neuroblastoma via unknown mechanisms in humans, and administration of the MRP1-selective inhibitor reversan (a lead compound of pyrazolopyrimidines, a prominent structural class of potent MRP1 inhibitors) suppressed growth of implanted neuroblastoma in mice. MRP3 mediates efflux transport of various endogenous compounds and metabolites, including leukotriene C4, glycocholic acid and estradiol glucuronide, from intracellular to extracellular space. Therefore, its inhibition by siRNA or inhibitor drugs may result in accumulation of various compounds in the intracellular space, and this in turn may result in cytotoxicity as shown in the present study. Although we demonstrated using mouse neuroblastoma that inhibition of Mrp3 by uricosuric drugs and...
NSAIDs induced cytotoxicity in the present study, MRP3 is also expressed in human neuroblastoma, and its transport activity can be similarly inhibited by uricosuric drugs and NSAIDs. Mouse and human MRP3 share more than 80% homology on a genetic level, and have several common substrates and inhibitors. Thus, MRPs are candidate targets for neuroblastoma treatment. However, further studies are needed to clarify the mechanism underlying the selective toxicity of MRP3 inhibition to neuroblastoma.

In this work, we established a fluorescence assay using Fluo-8 for screening Mrp3 inhibitors among candidate agents. Fluo-8 was simply used as a probe for evaluation of Mrp3 function, but not expected to induce cytotoxicity by its accumulation. Fluorescent dyes Fura-2 and Fluo-3 are transported by MRPs, but it is not yet established whether MRPs transport Fluo-8. We chose Fluo-8 for the screening system to detect Mrp3 inhibitors on the basis that it is a structural analog of Fluo-3 and its fluorescence intensity is about 4 times that of Fluo-3. Its suitability was confirmed by the observations that addition of the MRPs inhibitor probenecid or knockdown of Mrp3 with siRNA greatly increased the fluorescence intensity of Fluo-8 in Neuro2a cells preincubated with Fluo-8 AM (Figs. 2, 3). These results show that Mrp3 is a major efflux transporter of Fluo-8 in Neuro2a cells. This is interesting, because Fluo-8 is a cationic fluorescent dye, whereas Mrp3 preferentially transports organic acids.

Among inhibitors identified with the fluorescence assay, uricosuric drugs and NSAIDs induced a marked increase of fluorescence in Neuro2a cells, and we found a negative correlation ($r=−0.544$, $p<0.05$) between the fluorescence intensity of Fluo-8-loaded Neuro2a cells and MTT-reducing activity (Fig. 5). This result seems reasonable, because inhibition of Mrp function would lead to accumulation of cytotoxic products in these cells. Further screening for more potent inhibitors of Mrp3 might identify candidate therapeutic agents with even more selective cytotoxicity towards neuroblastoma. The strategy of high-throughput fluorescence screening using a transporter-specific fluorescent substrate might also be applicable to diseases other than neuroblastoma or cancer, if ABC xenobiotic transporters are possible molecular targets in those cases.

Probenecid is widely used in basic cytohistological experiments with the fluorescent dye Fura-2 to prevent leakage of the dye from the cells. This is consistent with the present observation that probenecid increases accumulation of Fluo-8 in Neuro2a cells (Fig. 3). In the present study, we found that several uricosuric drugs and NSAIDs inhibited efflux of Fluo-8 as potently as did 2.5 mM probenecid (Table 2). It has already been reported that these drugs inhibit MRP2 or MRP4. Also, NSAIDs such as salicylate, piroxicam, ibuprofen, naproxen, sulindac, tolmetin, etodolac, diclofenac, indomethacin, ketoprofen, phenylbutazone and celecoxib inhibit Mrp1, Mrp2 and/or Mrp4. Mrp2 and Mrp3 share 48% homology at the amino acid level, and have several common inhibitors. Thus, it is not unexpected that uricosuric drugs and NSAIDs also inhibit Mrp3 (Figs. 4, 6, Table 2). There is no report of side effects of uricosuric drugs on the nervous system, and this is consistent with the present finding that Mrp3 inhibition by uricosuric drugs may selectively suppress growth of neuroblastoma with minimal neuronal cytotoxicity. On the other hand, the pharmacological target of NSAIDs is cyclooxygenase (COX), and cytotoxicity of NSAIDs in Neuro2a cells may be partly caused by inhibition of this enzyme. However, NSAIDs at higher concentrations showed cytotoxicity to not only Neuro2a cells, but also primary cultured neurons (Fig. 6), in accordance with a previous report on neurotoxicity to hippocampal neurons, suggesting that inhibition of COX may induce nonspecific cytotoxicity. Thus, the present fluorescent screening system may also detect drugs with cytotoxicity via the mechanisms other than direct inhibition of Mrp3, but it would be beneficial as the first screening system to find candidate drugs which may possess selective cytotoxicity to neuroblastoma with minimal neurotoxicity.

In the present study, we searched for Mrp3 inhibitors among clinically used drugs. Such a drug-repositioning strategy can greatly reduce the cost of drug development. However, the cytotoxicity of the selected therapeutic drugs to Neuro2a cells was only observed at much higher concentrations than the clinically relevant ones. For example, the plasma unbound concentrations of probenecid, sulfinpyrazone, sulindac, mefenamic acid, flurbiprofen and phenylbutazone are 0.02–20 µM in the clinical situation (Table 3), whereas selective cytotoxicity in Neuro2a cells was only observed at concentrations over 100 or 1000 µM (Figs. 4, 6, Table 3). On the other hand, various types of chemical libraries are currently available, and they have been used to explore potent MRP inhibitors. Thus, further screening of high-affinity MRP3 inhibitors using larger chemical libraries may lead to the discovery of better candidates for agents with highly neuroblastoma-selective cytotoxicity.

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**Conflict of Interest** The authors declare no conflict of interest.

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