Mechanisms of glutathione-conjugate efflux from the brain into blood: Involvement of multiple transporters in the course

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Abstract

Accumulation of detrimental glutathione-conjugated metabolites in the brain potentially causes neurological disorders, and must therefore be exported from the brain. However, *in vivo* mechanisms of glutathione-conjugates efflux from the brain remain unknown. We investigated the involvement of transporters in glutathione-conjugates efflux using 6-bromo-7- ^{11}C methylpurine (^{11}C 1), which enters the brain and is converted into its glutathione conjugate, S-(7- ^{11}C methylpurin-6-yl)glutathione (^{11}C 2). In mice of control and knockout of P-glycoprotein/breast cancer resistance protein and multidrug resistance-associated protein 2 ($\text{Mrp2}^{-/-}$), ^{11}C 2 formed in the brain was rapidly cleared, with no significant difference in efflux rate. In contrast, ^{11}C 2 formed in the brain of $\text{Mrp1}^{-/-}$ mice was slowly cleared, whereas ^{11}C 2 microinjected into the brain of control and $\text{Mrp1}^{-/-}$ mice was 75% cleared within 60 min, with no significant difference in efflux rate. These suggest that Mrp1 contributes to ^{11}C 2 efflux across cell membranes, but not BBB. Efflux rate of ^{11}C 2 formed in the brain was significantly lower in $\text{Mrp4}^{-/-}$ and organic anion transporter 3 ($\text{Oat3}^{-/-}$) mice compared with control mice. In conclusion, Mrp1, Oat3, and Mrp4 mediate ^{11}C 2 efflux from the brain. Mrp1 may contribute to ^{11}C 2 efflux from brain parenchymal cells, while extracellular ^{11}C 2 is likely cleared across the BBB, partly by Oat3 and Mrp4.

Keywords

Blood–brain barrier, detoxification, efflux transporter, glutathione conjugate, positron emission tomography

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Introduction

Glutathione (GSH) conjugation plays an important role in detoxifying various endogenous and exogenous electrophiles and is typically catalysed by cytosolic GSH *S*-transferases.¹ In general, GSH conjugation renders electrophiles less reactive and toxic, although there are exceptions. For example, intracerebroventricular administration of a GSH conjugate of α -methyldopamine (a metabolite of the serotonergic neurotoxicants, 3,4-($\pm$)-(methylenedioxy)amphetamine [MDA] and 3,4-($\pm$)-(methylenedioxy)methamphetamine) to rats causes behavioural changes similar to those following subcutaneous administration of MDA.² In addition, human GSH *S*-transferases are inhibited by 5-*S*-glutathioinyl conjugates of dopamine and α -methyldopa.³

Regardless of whether GSH conjugates resulting from this process remain detrimental, they must be exported out of cells to be eliminated from the body.⁴ GSH conjugates are water-soluble and impermeable to the plasma membrane; therefore, their efflux from cells is mediated by efflux transporters such as multidrug resistance-associated protein 1 (MRP1) and MRP2.⁵

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Given that GSH conjugation occurs inside cells,¹ efflux of GSH conjugates from brain tissue is mediated by one or several efflux transporters in brain parenchymal cells, including neurons and non-neuronal cells, as well as efflux transporters at the abluminal and luminal side of brain capillary endothelial cells (BCECs). In the central nervous system, MRP1 is expressed at the blood–brain barrier (BBB) and blood–cerebrospinal fluid barrier.⁶ In the adult and developing human brain, MRP1 is also expressed in nonendothelial cells such as astrocytes and neurons.^{7,8} P-glycoprotein and breast cancer resistance protein (BCRP) are major efflux transporters at the BBB, while MRP2 may also be expressed at the BBB.⁹ In addition, MRP4 is an efflux transporter expressed in the brain, with various anionic compounds including conjugates being substrates for MRP4.^{10,11} The interplay between organic anion transporter 3 (OAT3) and MRP4 at the BBB may enable vectorial transport of prostaglandins across endothelial cells.¹² Based on these findings, candidate transporters for GSH-conjugate efflux from the brain include MRP1, MRP2, MRP4, OAT3, P-glycoprotein, and BCRP. However, in vivo mechanisms of GSH-conjugate efflux from the brain remain unknown. Identification of these mechanisms would facilitate understanding of the pathogenesis of neurological disorders, which are potentially caused by accumulation of toxic brain metabolites.

Because of methodological difficulties, in vivo measurement of efflux transport has been challenging, particularly in the brain. The BBB prevents delivery of most hydrophilic probes/tracers for efflux measurement into the brain by intravenous administration. Consequently, a method that overcomes this limitation and allows GSH-conjugate efflux from the brain to be noninvasively assessed was developed.¹³ Accordingly, the positron emission tomography (PET) tracer, 6-bromo-7-^[11C]methylpurine (^[11C]1), was designed based on this method. Because of its lipophilicity, ^[11C]1 can enter the brain after intravenous injection and become converted into its GSH conjugate, S-(7-^[11C]methylpurin-6-yl)glutathione (^[11C]2).¹⁴ Thus, ^[11C]1 enables assessment of GSH-conjugate efflux from the brain noninvasively, with the efflux rate of ^[11C]2 apparently reflecting MRP1 activity.¹⁴ However, ^[11C]1 cannot distinguish between GSH-conjugate efflux from brain parenchymal cells and through the BBB (Figure 1(a), left). In this study, we investigated the involvement of candidate transporters in ^[11C]2 efflux from the brain using ^[11C]1 and PET. The contribution of Mrp1 at the BBB to ^[11C]2 efflux was also investigated using the intracerebral injection method (Figure 1(a), right). This is similar to the brain efflux index (BEI), which has been proposed as a novel approach for analysing efflux at the BBB.¹⁵

Materials and methods

All experiments were approved by the National Institute of Radiological Sciences (Chiba, Japan). 6-Bromo-7-^[11C]methylpurine (^[11C]1) was synthesised as described previously.¹⁴ S-(7-^[11C]methylpurin-6-yl)glutathione (^[11C]2) was manually synthesised as described previously.¹⁶

Animals

The animal experimental protocol was approved by the Animal Care and Use Committee of the National Institute of Radiological Sciences (Permit Number: 16-1006). All animal experiments have been reported following ARRIVE (Animal Research: Reporting of In vivo Experiments) guidelines and were conducted in accordance with the Guidelines Regarding Animal Care and Handling of the National Institute of Radiological Sciences. Investigators were not blinded to genotype when assessing outcomes, but brain radioactivity was measured by PET and efflux rates were determined objectively (by fitting data to the monoexponential function), thereby reducing the potential for investigator bias. PET enabled a reduction in the number of mice required for a given experiment, as experiments were performed using a single animal.

Male FVB/NJcl mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). Male *Mrp1*^{−/−} mice, *Mrp2*^{−/−} mice, and *Mdr1a/b*^{−/−}*Bcrp*^{−/−} triple-knock-out mice (which lack the genes for *Mdr1a/1b* and *Bcrp*) were purchased from Taconic Farms, Inc. (Hudson, NY, USA). *Oat3*^{+/−} and *Mrp4*^{+/−} mice were obtained from Deltagen, Inc. (San Mateo, CA, USA) and Trans Genic Inc., Ltd (Kumamoto, Japan), respectively. To increase the number of heterogeneous mice, mice were mated with C57BL/6J mice (Charles River Laboratories Japan, Inc., Kanagawa, Japan). First-generation homozygous mice (*Oat3*^{−/−} and *Mrp4*^{−/−}) were generated from corresponding heterogeneous mice, followed by breeding of *Oat3*^{−/−} and *Mrp4*^{−/−} mice at Charles River Laboratories Japan, Inc. Male *Oat3*^{−/−} and *Mrp4*^{−/−} mice were purchased from Charles River Laboratories Japan, Inc. All mice were maintained in cages (maximum five per cage) with bedding materials, a mixture of ALPHA-dri and Shepherd's Cob (EPS Ekishin Co., Ltd, Tokyo, Japan), under conventional temperature, humidity, and light conditions, where they were allowed free access to food and water. *Mrp1*^{−/−} (15–21 weeks, 30–33 g), *Mrp2*^{−/−} (12–16 weeks, 29–33 g), *Mdr1a/b*^{−/−}*Bcrp*^{−/−} (14–16 weeks, 23–33 g), *Mrp4*^{−/−} (8–18 weeks, 18–30 g), and *Oat3*^{−/−} (8–9 weeks, 20–22 g) mice were used in this study. FVB/NJcl mice (8–19 weeks, 27–35 g) were used as controls for *Mrp1*^{−/−},

Figure 1. (a) Diagrammatic representation of clearance of the glutathione conjugate, S-(7-[^{11}C]methylpurin-6-yl)glutathione ($[^{11}\text{C}]\mathbf{2}$), from the brain. (a, left) Intravenous injection of 6-bromo-7-[^{11}C]methylpurine ($[^{11}\text{C}]\mathbf{1}$). [$^{11}\text{C}]\mathbf{1}$ penetrates the blood–brain barrier and undergoes rapid conversion to [$^{11}\text{C}]\mathbf{2}$ within brain capillary endothelial cells (BCECs) and brain parenchymal cells. [$^{11}\text{C}]\mathbf{2}$ formed within brain parenchymal cells must penetrate multiple membranes to be eliminated from the brain into blood, namely, parenchymal cell membranes and abluminal and luminal membranes of BCECs. (a, right) Intracerebral injection of [$^{11}\text{C}]\mathbf{2}$. Intracerebrally injected [$^{11}\text{C}]\mathbf{2}$ penetrates both BCEC membranes to be cleared from the brain into blood. (b) Structures of [$^{11}\text{C}]\mathbf{1}$ and [$^{11}\text{C}]\mathbf{2}$.

Mrp2^{−/−}, and *Mdr1a/b*^{−/−}*Bcrp*^{−/−} mice, while C57BL/6J mice (11–13 weeks, 23–27 g) were used as controls for *Mrp4*^{−/−} and *Oat3*^{−/−} mice. Mice were anesthetised using 1%–2% isoflurane and a small animal anaesthetic (TK-6; Bio Machinery, Chiba, Japan), because isoflurane has a safety profile and it is easy to regulate the depth and duration of anaesthesia. All animal experiments were performed during the daytime, and the order in which mice were tested was randomly chosen.

PET scanning procedures

PET scanning procedures were performed using an Inveon Dedicated PET System (Siemens Medical

Solutions, Knoxville, TN, USA) in the same manner as described previously.¹⁴

Intravenous injection of [$^{11}\text{C}]\mathbf{1}$. A total of 28 mice ($n=4$ for each of the following seven groups: FVB/NJcl, *Mrp1*^{−/−}, *Mrp2*^{−/−}, *Mdr1a/b*^{−/−}*Bcrp*^{−/−}, C57BL/6J, *Mrp4*^{−/−}, and *Oat3*^{−/−}) were used for scanning. In brief, [$^{11}\text{C}]\mathbf{1}$ (10–15 MBq; <0.5 nmol) was intravenously administered to each mouse through a lateral tail vein. PET data were acquired for 60 min after injection. Volumes of interest were manually placed on the whole brain, and time–radioactivity concentration (Bq/mL) curves were generated using ASIPro VM software (CTI Concorde Microsystems, Knoxville, TN, USA). The efflux rate (min^{-1}) of [$^{11}\text{C}]\mathbf{2}$ generated in the

brain was determined by fitting time–radioactivity curves to the monoexponential function.¹⁴

Intravenous injection of [¹¹C]2. Six mice (FVB/NJcl and *Mrp1*^{−/−}; *n*=3 each) were used for scanning. Tracer [¹¹C]2 (12–13 MBq) was intravenously administered to each mouse through the lateral tail vein, and time–radioactivity concentration (Bq/mL) curves generated as described above.

Intracerebral microinjection of [¹¹C]2. A total of 16 mice were used (FVB/NJcl and *Mrp1*^{−/−}; *n*=8 each). Intracerebral injection was performed using the BEI method^{15,17} with some modifications. The BEI method requires a reference compound (e.g. inulin) for correction of interindividual differences in injected doses. However, the kinetics of intracerebrally-injected [¹¹C]2 in mouse brain can be sequentially monitored by PET, and thus the reference compound was not necessary for this PET study. Kinetics of [¹¹C]1 in the brain of eight *Mrp1*^{−/−} mice was assessed one to two weeks prior to this experiment. Accordingly, efflux rates in all *Mrp1*^{−/−} mice were shown to be reduced by approximately 90%. Mice were anaesthetised with isoflurane throughout the experiment. After exposure of the skull, two small holes were made in the skull (± 3.5 mm lateral to bregma) using a dental drill. The injection site was determined using a stereotaxic frame. A microinjection needle (inner diameter, 0.13 mm; outer diameter, 0.3 mm) was inserted into each hole to a depth of 2.5 mm. [¹¹C]2 solution (44–111 kBq, <3.7 pmol) was simultaneously injected into the left and right hemispheres of the brain over a period of 4 min using a constant infusion pump (Model ESP-64; Eicom Corporation, Kyoto, Japan). The volume injected into the brain was calculated to be 0.55 μ L (mean) from the residual tube volume. After injection, the needle was kept in place for 3 min and then withdrawn. Mice were immediately transported to the scanner bed and scanned by PET. Computed tomography (CT) images were also acquired with an X-ray source set at 80 kVp and 500 μ A using an Inveon CT scanner (Siemens Medical Solutions). PET and CT images were fused using Inveon Research Workplace software (Siemens Medical Solutions). Volumes of interest were manually placed on the injection site, and time–radioactivity concentration (Bq/mL) curves generated using ASIPro VM software (CTI Concorde Microsystems, Knoxville, TN, USA). Efflux rate (min^{-1}) of [¹¹C]2 injected into the brain was determined by fitting time–radioactivity curves (0–60 min) to the monoexponential function, as described previously.¹⁴

When [¹¹C]2 was successfully injected into both right and left sides of the mouse brain, efflux rates were

measured on each side, and the averaged value used as data for each mouse. When [¹¹C]2 was injected into either the right or left side of the mouse brain, the efflux rate determined on one side was used as data for each mouse.

Statistical analysis

No statistical methods were used to predetermine sample size. Data distribution was assumed to be normal, but this was not formally tested. Statistical analysis was performed using R (version 3.3.2; R Foundation for Statistical Computing, Vienna, Austria). Differences between two groups were analysed by two-tailed unpaired Student's *t*-test, while Dunnett's test was used for multiple comparisons. Significance level was set at 0.05 for all analyses.

Results

Kinetics of [¹¹C]2 formed from [¹¹C]1 in the brain of control, *Mrp1*^{−/−}, *Mrp2*^{−/−}, and *Mdr1a/b*^{−/−}*Bcrp*^{−/−} mice

Figure 2(a) and (b) shows time–radioactivity curves in the brain after intravenous injection of [¹¹C]1 into control and knockout mice. The kinetics were similar in *Mrp2*^{−/−} and *Mdr1a/b*^{−/−}*Bcrp*^{−/−} mice to control mice, whereas radioactivity clearance in *Mrp1*^{−/−} mice was delayed. Efflux rates (mean $\pm$ standard deviation [SD]) were as follows (Figure 2(c)): $0.023 \pm 0.0016 \text{ min}^{-1}$ (control), $0.0024 \pm 0.00027 \text{ min}^{-1}$ (*Mrp1*^{−/−}), $0.025 \pm 0.00066 \text{ min}^{-1}$ (*Mrp2*^{−/−}), and $0.024 \pm 0.00070 \text{ min}^{-1}$ (*Mdr1a/b*^{−/−}*Bcrp*^{−/−}). There was a significant difference in efflux rate between control and *Mrp1*^{−/−} mice (Dunnett's test, *P* < 0.05) but not *Mrp2*^{−/−} (*P* = 0.19) or *Mdr1a/b*^{−/−}*Bcrp*^{−/−} mice (*P* = 0.76).

Brain uptake following intravenous injection of [¹¹C]2 into control and *Mrp1*^{−/−} mice

To confirm that [¹¹C]2 cannot enter the brain of either control or *Mrp1*^{−/−} mice, brain uptake of [¹¹C]2 was measured following intravenous injection of [¹¹C]2 (Figure 3). No difference in brain kinetics of [¹¹C]2 was observed between control and *Mrp1*^{−/−} mice. Radioactivity levels in the brain of control and *Mrp1*^{−/−} were extremely low and <1% injected dose (ID)/mL for 60 min after intravenous injection.

Brain kinetics following intracerebral injection of [¹¹C]2 in control and *Mrp1*^{−/−} mice

Typical PET images are shown in Figure 4(a). Radioactivity was observed not only in the injection

Figure 2. (a) Time–radioactivity curves for the brain obtained by positron emission tomography after intravenous administration of 6-bromo-7- $[^{11}\text{C}]$ methylpurine ($[^{11}\text{C}]\mathbf{1}$) into control and knockout mice. Values were normalised to the highest value, which was taken as 100%, and expressed as mean $\pm$ SD ($n = 4$ per group). (b) Time–radioactivity curves for the period of 15 to 60 min after intravenous injection. The Y-axis is presented on a logarithmic scale. Regression curves based on first-order kinetics are also shown. (c) Efflux rates of S-(7- $[^{11}\text{C}]$ methylpurin-6-yl)glutathione ($[^{11}\text{C}]\mathbf{2}$) formed in the brain after intravenous administration of $[^{11}\text{C}]\mathbf{1}$. ns: not significant. * $P < 0.05$, compared with controls (Dunnett's test). $n = 4$ per group.

site but also the bladder. This demonstrates that intracerebrally injected $[^{11}\text{C}]\mathbf{2}$ is transported from the brain to the circulatory system, and finally excreted into urine. The kinetics were very similar in *Mrp1*^{-/-} and control mouse brain, with brain radioactivity decreasing over the 60-min observation period (Figure 4(b)). Efflux rates (mean $\pm$ SD) of $[^{11}\text{C}]\mathbf{2}$ in control and *Mrp1*^{-/-} mice were $0.024 \pm 0.0080 \text{ min}^{-1}$ and $0.020 \pm 0.0082 \text{ min}^{-1}$, respectively (Figure 4(c)). There was no significant difference in efflux rate between control and *Mrp1*^{-/-} mice (Student's t -test, $P = 0.27$).

Kinetics of $[^{11}\text{C}]\mathbf{2}$ formed from $[^{11}\text{C}]\mathbf{1}$ in the brain of control, *Mrp4*^{-/-}, and *Oat3*^{-/-} mice

Figure 5(a) and (b) shows time–radioactivity curves in the brain after intravenous injection of $[^{11}\text{C}]\mathbf{1}$ into control, *Mrp4*^{-/-}, and *Oat3*^{-/-} mice. Radioactivity clearance was delayed in the brain of *Mrp4*^{-/-} and *Oat3*^{-/-} mice. Efflux rates (mean $\pm$ SD) were as follows (Figure 5(c)): $0.024 \pm 0.0010 \text{ min}^{-1}$ (control), $0.015 \pm 0.0016 \text{ min}^{-1}$ (*Mrp4*^{-/-}), and $0.0088 \pm 0.0010 \text{ min}^{-1}$ (*Oat3*^{-/-}). Significant differences in efflux rates were observed between control and *Mrp4*^{-/-} mice

Figure 3. Positron emission tomography images after intravenous administration of S-(7-[^{11}C]methylpurin-6-yl)glutathione (^{11}C]2) into control and *Mrp1* $^{-/-}$ mice. Summed images at 0–60 min after administration are shown.

(Dunnett's test, $P < 0.05$) and between control and *Oat3* $^{-/-}$ mice (Dunnett's test, $P < 0.05$).

Discussion

Previous studies have demonstrated the following: (1) [^{14}C]1 exhibits high reactivity with GSH in mouse brain homogenates¹⁸; (2) GSH conjugate resulting from the reaction is a substrate for MRP1 in an *in vitro* system using inside-out membrane vesicles¹³; (3) after intravenous injection, [^{14}C]1 is converted to GSH conjugate in the brain of *Mrp1* $^{-/-}$ and wild-type mice by 15 min¹⁴; (4) therefore, clearance rate of brain radioactivity at 15–60 min after [^{11}C]1 injection reflects the efflux rate of GSH conjugate (^{11}C]2) from brain; and (5) efflux rate is approximately 10-fold lower in *Mrp1* $^{-/-}$ mice than wild-type mice.¹⁴ These findings suggest that [^{11}C]2 is exclusively transported from the brain by MRP1. Indeed, this assumption is further supported by our present finding that neither Mrp2, P-glycoprotein, nor Bcrp affect the efflux rate of [^{11}C]2 formed in the brain (Figure 2). In addition, influx of [^{11}C]2 from blood to brain in control and *Mrp1* $^{-/-}$ mice was extremely low, even negligible for a period of 60 min after intravenous injection (Figure 3), indicating that [^{11}C]2 in blood cannot cross the BBB. The efflux rate of [^{11}C]2 from the brain of various knockout mouse lines is summarised in Figure 6.

A recent study has shown that MRP4 contributes to elimination of brain radioactivity after [^{11}C]1 injection into mice.¹⁹ In addition, we found that Mrp4 and Oat3 also contribute to elimination of [^{11}C]2 formed in the

brain (Figure 5). Reduction in efflux rate in *Mrp1* $^{-/-}$ mice suggests that other transporters contribute to approximately 10% of the efflux of [^{11}C]2 formed in the brain (Figure 2). However, the contribution of Mrp4 (38%) and Oat3 (63%) exceeds 10%, suggesting that Mrp1 is involved in [^{11}C]2 efflux at a site distinct from Mrp4 and Oat3. The interplay between Oat3 and Mrp4 at the BBB is reported to enable vectorial transport of prostaglandins from the brain interstitial fluid to the blood across BCECs.¹² In mice, Oat3 is localised at the abluminal membrane of BCECs,²⁰ whereas Mrp4 is localised at the luminal membrane of these cells.²¹ In other words, Oat3 transports prostaglandin from the interstitial fluid into BCECs, and then Mrp4 extrudes it from BCECs into the blood. Similar to prostaglandins, [^{11}C]2 in the extracellular space may be taken up into BCECs by Oat3 and then pumped out of these cells into the blood by Mrp4 to be eliminated from the brain. Nonetheless, we cannot exclude the possibility that other transporters contribute to uptake and efflux processes in BCECs.

The tracer [^{11}C]1 can readily enter brain tissue because of its lipophilicity, and therefore should be able to diffuse into all brain cells, including BCECs and brain parenchymal cells (neurons and non-neuronal cells). In addition, GSH S-transferases are found in neurons, glial cells, and endothelial cells in the rodent brain.^{22–24} Given these findings, formation of [^{11}C]2 would occur within BCECs and brain parenchymal cells. To investigate the *in vivo* contribution of Mrp1 at the BBB to [^{11}C]2 efflux, we used an intracerebral injection method (similar to the BEI method), which has been proposed as a novel approach for analysing efflux at the BBB.¹⁵ Here, we found no significant difference in efflux rate between control and *Mrp1* $^{-/-}$ mice (Figure 4), and [^{11}C]2 was eliminated from the brain, likely through the BBB. Alternatively, [^{11}C]2 injected into the extracellular space might be cleared via convective (bulk) flow or the glymphatic system, a system of cerebrospinal fluid–interstitial fluid exchange that promotes clearance of waste products and metabolites from the brain.^{25,26} In any case, Mrp1 does not appear to contribute to clearance of intracerebrally injected [^{11}C]2. Furthermore, [^{11}C]2 formed in the brain (within cells) after [^{11}C]1 injection was not cleared in *Mrp1* $^{-/-}$ mice (Figure 2). These findings suggest that Mrp1 in brain parenchymal cells, but not at the BBB, is involved in [^{11}C]2 efflux from the brain. In control mice, efflux rates of [^{11}C]2 for intracerebral and intravenous injections were comparable, and consequently it is difficult to evaluate Mrp1 activity in the mouse brain using [^{11}C]1, unless there are large changes in Mrp1 activity, such as in gene knockout animals.

Figure 4. (a) Typical positron emission tomography (PET) image fused with X-ray computed tomography image after intracerebral injection of S-(7-[¹¹C]methylpurin-6-yl)glutathione ([¹¹C]2) into a control mouse. Summed images (0–60 min after injection) are shown. (b, left) Time–radioactivity curves for the brain obtained by PET after intracerebral injection of [¹¹C]2 into *Mrp1*^{-/-} and control mice. Values were normalised to the highest value, which was taken as 100%, and expressed as mean ± SD (*n* = 8 per group). (b, right) Time–radioactivity curves after intracerebral injection. The Y-axis is presented on a logarithmic scale. Regression curves based on first-order kinetics are also shown. (c) Efflux rates of [¹¹C]2 injected into the brain of *Mrp1*^{-/-} or control mice. There was no significant difference in efflux rate between the two groups (Student's *t*-test, *P* = 0.27, *n* = 8 per group). Kinetics of 6-bromo-7-[¹¹C]methylpurine ([¹¹C]1) in the brain of eight *Mrp1*^{-/-} mice was assessed one to two weeks prior to intracerebral injection of [¹¹C]2. Efflux rate in all *Mrp1*^{-/-} mice was reduced by approximately 90%.

Figure 5. (a) Time–radioactivity curves for the brain obtained by positron emission tomography after intravenous administration of 6-bromo-7- $[^{11}\text{C}]$ methylpurine ($[^{11}\text{C}]\mathbf{1}$) into control and knockout mice. Values were normalised to the highest value, which was taken as 100%, and expressed as mean $\pm$ SD ($n = 4$ per group). (b) Time–radioactivity curves for the period of 15 to 60 min after intravenous injection. The Y-axis is presented on a logarithmic scale. Regression curves based on first-order kinetics are also shown. (c) Efflux rates of S-(7- $[^{11}\text{C}]$ methylpurin-6-yl)glutathione ($[^{11}\text{C}]\mathbf{2}$) formed in the brain after intravenous administration of $[^{11}\text{C}]\mathbf{1}$. * $P < 0.05$, compared with controls (Dunnett's test). $n = 4$ per group.

Indeed, heterozygous *Mrp1*^{+/-} mice, which are expected to have a 50% reduction in Mrp1 expression, are reported to have only a moderate reduction in the elimination rate of brain radioactivity after $[^{11}\text{C}]\mathbf{1}$ injection compared with wild-type mice.¹⁹ This implies that the transport system is very high capacity, i.e. the high ratio of maximal transport rate (T_{max}) to the Michaelis–Menten constant (K_m).

Clearance rate of an inert polar compound that is neither transported across the BBB nor retained by the brain can provide a measure of interstitial fluid bulk flow (convective flow), and indeed, inulin has been used for this purpose.²⁷ If $[^{11}\text{C}]\mathbf{2}$ uptake from the extracellular space into BCECs by transporters is completely

blocked, convective flow should dominate $[^{11}\text{C}]\mathbf{2}$ efflux from the brain because hydrophilic $[^{11}\text{C}]\mathbf{2}$ cannot cross membranes via simple diffusion (Figure 3). In the rat brain, efflux rates for a wide range of water-soluble compounds, including inulin, are reported to be between 0.21 and 0.59 h⁻¹ (i.e. 0.0035 and 0.0098 min⁻¹).²⁸ Clearance rate of inulin in awake mice is roughly 0.006 min⁻¹, although it is increased by anaesthesia with ketamine/xylazine.²⁷ In this study, the efflux rate of $[^{11}\text{C}]\mathbf{2}$ in the brain of *Oat3*^{-/-} mice was 0.0088 min⁻¹. Interestingly, efflux rates of structurally unrelated anionic compounds in the brain of *Oat3*^{-/-} mice are approximately 20% of control mice, and similar to $[^{11}\text{C}]\mathbf{2}$: 0.013 min⁻¹ for ¹¹C-hippuric acid

Figure 6. Schematic summary of the efflux rates of S-(7-[^{11}C]methylpurin-6-yl)glutathione ([^{11}C]2).

and 0.0089 min^{-1} for a metabolite of $^{99\text{m}}\text{Tc}$ complex with *N,N'*-1,2-ethylenediylbis-L-cysteine diethyl ester ($^{99\text{m}}\text{Tc}$ -ECD).^{29,30} Therefore, [^{11}C]2 clearance in *Oat3*^{-/-} mouse brain appears to be accomplished by convective flow rather than transporters. In humans, GSH conjugates extruded from brain parenchymal cells to the extracellular space by MRP1 might be cleared by bulk flow or other uptake transporters, as OAT3 is poorly expressed in BCEC in primates, including humans, but highly expressed in mice.³¹

In vivo function of MRP1 and its location at the BBB remains controversial. Using an in situ brain perfusion technique, previous studies have suggested that Mrp1 has no functional effect at the luminal side of the BBB.^{32,33} This is consistent with our results showing that Mrp1 does not contribute to [^{11}C]2 efflux at the BBB. In contrast, Mrp1 is involved in the elimination of 17 β -estradiol-D-17 β -glucuronide (E_2 17 β G) at the BBB, as shown using the BEI method.¹⁷ The reported elimination rate constants for [^3H] E_2 17 β G in wild-type and *Mrp1*^{-/-} mice were 0.007 and 0.004 min^{-1} , respectively. We cannot completely exclude the possibility of a role for Mrp1 at the BBB in mouse brain. However, in vivo contribution of Mrp1 at the BBB to E_2 17 β G efflux appears minimal because the efflux rates are within the range of convective efflux (0.0035 – 0.0098 min^{-1}),²⁸ although no significant amount of E_2 17 β G was found in the contralateral cerebrum, cerebellum or cerebrospinal fluid compartment.¹⁷ Mrp1 at the abluminal side of the BBB is also reported to promote accumulation of compounds in the brain.³⁴ The reason for this discrepancy with our results remains unknown. The rate of efflux of [^{11}C]2 by abluminal Mrp1 in BCECs to the extracellular space might be much lower than the rate of influx by abluminal Oat3 from the extracellular space into BCECs.

Conclusion

Three transporters, Mrp1, Oat3, and Mrp4, mediate efflux of the GSH conjugate [^{11}C]2 from the brain. Mrp1 may be involved in [^{11}C]2 efflux from brain parenchymal cells, while [^{11}C]2 in the extracellular space of the brain is likely cleared in part by transport across the BBB by the combination of Oat3 and Mrp4. Convective efflux might also contribute to [^{11}C]2 clearance from the extracellular space.

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Authors' contributions

TO designed the research; TO, MO, and HW performed the research; TO, MO, TK, HW, and MRZ analysed data and wrote the paper. All authors read and approved the final manuscript.

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