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A novel noninvasive method for assessing glutathione-conjugate efflux systems in the brain

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Abstract—Brain efflux systems export such conjugated metabolites as glutathione (GSH) and glucuronate conjugates, generated by the detoxification process, from the brain and serve to protect the brain from harmful metabolites. The intracerebral injection of a radiolabeled conjugate is a useful technique to assess brain efflux systems; however, this technique is not applicable to humans. Hence, we devised a novel noninvasive approach for assessing GSH-conjugate efflux systems using positron emission tomography. Here, we investigated whether or not a designed proprobe can deliver its GSH conjugate into the brain. Radiolabeled 6-chloro-7-methylpurine (7m6CP) was designed as the proprobe, and [^{14}C]7m6CP was prepared by the reaction of 6-chloropurine with [^{14}C]CH_3I as a model of [^{11}C]CH_3I. The radiochemical yield and purity of [^{14}C]7m6CP were 10–20% and greater than 99%, respectively. High brain uptake (0.8% ID/g) at 1 min was observed, followed by gradual radioactivity clearance from the brain for 5–60 min after the injection of [^{14}C]7m6CP into rats. Analysis of metabolites confirmed confirmed that the presence of [^{14}C]7m6CP was hardly observed, and 80% of the radioactivity was identical to its GSH conjugate for 15–60 min. The brain radioactivity was single-exponentially decreased during the period of 15–60 min post-injection of [^{14}C]7m6CP, and the first-order efflux rate constant of the conjugate, estimated from the slope, was 0.0253 min^{-1}. These results showed that (1) [^{14}C]7m6CP readily entered the brain, (2) it efficiently and specifically transformed to the GSH conjugate within the brain, and (3) after [^{14}C]7m6CP disappearance, the clearance of radioactivity represented the only efflux of GSH conjugate. We conclude that 7m6CP can deliver the GSH conjugate into the brain and would be useful for assessing GSH-conjugate efflux systems noninvasively.

1. Introduction

There are active efflux transporters at the blood–brain barrier (BBB) and/or blood–cerebrospinal fluid barrier, and they can export potentially harmful metabolites and xenobiotics from the brain.[1,2] Hydrophilic conjugated metabolites such as glutathione (GSH) and glucuronate conjugates, generated by detoxification of xenobiotics, are also eliminated from the brain via efflux systems.[3] Currently, these conjugates are known to be good substrates for the multidrug resistance-associated protein (MRP) family involved in brain efflux systems.[4,5] Sultana and Butterfield observed a slight increase in MRP1 protein expression and adducts MRP1 bound to 4-hydroxy-2-transnonenal (a lipid peroxidation product) in frozen hippocampal samples from Alzheimer's patients versus age-matched control subjects.[6] Several therapeutic drugs such as probenecid and indomethacin are known to be a MRP1 inhibitor.[7,8] A noninvasive assessment of brain efflux systems in human studies will be useful for elucidating pathological conditions of neurodegenerative disorders, and directly evaluating the pharmacological effect of therapeutic drug on brain efflux systems. The efflux rate constant, reflecting brain efflux systems, can be calculated from a time course measurement of brain concentration following the intracerebral injection of a conjugate. Indeed, using the conjugate of radiolabeled 17β-estradiol-D-17β-glucuronide ($E_2$17βG), efflux systems involved in the transport of $E_2$17βG across the BBB were investigated.[9] The intracerebral injection of a radiolabeled conjugate is a useful technique to study mechanisms of brain-to-blood efflux;

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however, this invasive method is not applicable to human study. We therefore devised a novel method, referred to as the Metabolite Extrusion Method (MEM), for the noninvasive assessment of GSH-conjugate efflux systems in living human brains using positron emission tomography (PET). Positron emitters are not convenient to perform basic experiments because of a very short half-life. Here, we studied whether or not proprobes designed according to MEM can noninvasively deliver their GSH conjugate into the brain using rats and ^{14}C nuclide (half-life: 5730 yr) in place of ^{11}C nuclide for PET (half-life: 20.4 min).

2. Results and discussion

2.1. Design and chemistry

The rationale for MEM is illustrated in Figure 1a and b. A radiolabeled lipophilic compound, a proprobe, is administered intravenously. After injection, the proprobe enters the brain across the BBB by simple diffusion (K_1). While a portion of the incorporated proprobe diffuses back into the blood (k_2), the rest is rapidly converted to the probe, GSH conjugate (k_3). If the conversion in the peripheral tissues is also rapid, the proprobe no longer exists in either the blood or the brain with a short lag time after the injection, in which case the situation of Figure 1a changes to that of Figure 1b. If passive transport of the GSH conjugate across the BBB is negligible, the efflux rate of the GSH conjugate (k_{eff}) can be estimated noninvasively. MEM also provides an accurate k_{eff} because of the simple parameter estimation of the single exponential curve, as cerebral blood flow can be measured by the accurate estimation of the clearance rate of brain radioactivity following the administration of radioactive xenon.[10,11] MEM has the merit that k_{eff} can be estimated without the need for arterial blood sampling, which requires considerable technical expertise.

Our goal is to deliver a GSH conjugate into the brain for the noninvasive assessment of GSH-conjugate efflux systems. To achieve this, the proprobe must possess three properties: high BBB permeability and efficient conversion into the single probe (permeability, efficiency, and specificity). The probe should not penetrate the BBB by simple diffusion. Furthermore, it is desirable that the proprobe platform can be easily modified for the introduction of a lipophilic group and radiolabeling. 6-Chloropurine is reported to be converted to *S*-(6-purinyl)glutathione by hepatic and renal glutathione *S*-transferase (GST) in vivo.[12] The rat brain has a GSH concentration of approximately 2 mM, and GST is markedly expressed in the brain.[13,14] The adaptation of purine as a proprobe platform allows easy chemical modification. Given these aspects, we selected 6-chloropurine as the basic skeleton of the proprobe. The methylation of 6-chloropurine with [^{14}C]CH_3I as a model for [^{11}C]CH_3I was performed to increase lipophilicity and introduce radioactivity. As a consequence, two potential proprobes for the delivery of a GSH conjugate into the brain were generated: 6-chloro-9-[^{14}C]methylpurine ([^{14}C]9m6CP) and 6-chloro-7-[^{14}C]methylpurine

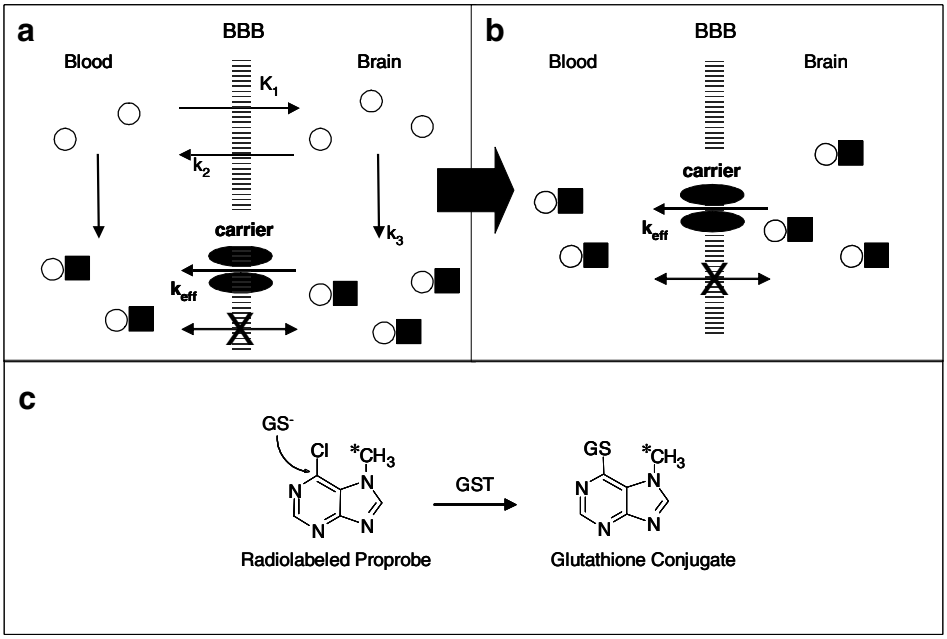

Figure 1. Diagrammatic representation of the Metabolite Extrusion Method (a and b). A proprobe (open circles) is intravenously administered. The proprobe can penetrate the BBB and transform to a probe of GSH conjugate (circle-squares) in the brain. The probe is extruded by efflux systems from the brain to the blood. After the proprobe is completely converted to the probe and no longer exists in either the brain or the blood, the situation of Fig. 1a changes to that of Fig. 1b, similar to the state obtained by intracerebral injection. K_1 and k_2 are the rate parameters illustrating transport of the proprobe across the BBB by diffusion, while k_3 and k_{eff} are the rate parameters indicating the GSH conjugation and transport of the probe by efflux systems, respectively. A proprobe designed to deliver the radiolabeled GSH conjugate into the brain (c). Of two isomers generated by the methylation of 6-chloropurine, 6-chloro-7-methylpurine is shown in the figure. GS^- denotes the thiolate anion of GSH.

([^{14}C]7m6CP) (Fig. 1c). Radiochemical yield and purity of [^{14}C]9m6CP were 50% and 99%, respectively. Those of [^{14}C]7m6CP were 10–20% and 99%, respectively. The production rate for isomers is similar to an early report that the alkylation of 6-chloropurine with alkyl halide formed major products (9-isomers) and minor products (7-isomers).[15] The steric hindrance between chlorine atom and methyl group probably leads to the reduction of [^{14}C]7m6CP formation.

2.2. Verification of requirements (permeability, efficiency, and specificity)

For reliable estimation of k_{eff}, each proprobe should possess physiochemical properties required for MEM: permeability, efficiency, and specificity. Figure 2a shows brain uptake curves following the intravenous injection of [^{14}C]7m6CP and [^{14}C]9m6CP into rats. They exhibit a similar high uptake (0.7–0.8% ID/g) at 1 min after injection, and then a gradual decrease in brain radioactivity from 5 min. In general, the delivery of a low molecular substance into the brain is not so difficult as long as that substance is a lipophilic one. Indeed, these proprobes showed a similar high uptake at 1 min after injection, which is comparable to the 1 min uptake of a clinically used radiopharmaceutical,[16] and they had the high BBB permeability. However, the two proprobes showed quite different kinetics thereafter: while [^{14}C]9m6CP exhibited a gradual elimination of radioactivity, [^{14}C]7m6CP displayed clearance after temporary accumulation. This is likely due to the enzymatic conversion rates of two proprobes to GSH conjugates via GST in the brain, that is, k_3 in Figure 1a. In order to examine this, the reaction rates were determined in rat brain homogenates.

Figure 3a shows the first-order rate constant (in vitro k_3) at a unit concentration of brain tissue. The rates of [^{14}C]7m6CP and [^{14}C]9m6CP were estimated as 0.33 ± 0.01 and 0.0057 ± 0.0002 ($min^{-1}\ g^{-1}\ mL^{-1}$), respectively. To confirm that proprobes are metabolized in the brain according to the in vitro k_3, the fraction of each unchanged form in the brain was analyzed 15 min after intravenous injection using thin layer chromatography (TLC). The radioactivity corresponding to the unchanged form almost completely disappeared at 15 min post-injection of [^{14}C]7m6CP, while for [^{14}C]9m6CP, approximately 80% of radioactivity remained in the unchanged form (Fig. 3b). The in vivo results could be reasonably explained by differences in the in vitro k_3 between [^{14}C]7m6CP and [^{14}C]9m6CP. [^{14}C]9m6CP would mostly diffuse back into the blood without reaction with GSH because of its slower reaction rate. In contrast, [^{14}C]7m6CP (high in vitro k_3) appears to be rapidly converted to a radioactive metabolite, which is subsequently extruded from the brain to the blood. In other words, the decrease in radioactivity following the injection of [^{14}C]9m6CP implies mainly the washout of the intact proprobe from the brain, whereas that of [^{14}C]7m6CP means the washout of the radioactive metabolite. As [^{14}C]9m6CP cannot sufficiently deliver the GSH conjugate into the brain, it is abandoned as a proprobe for MEM in this study.

[^{14}C]7m6CP readily entered the brain, and it was rapidly metabolized within the brain. Further analysis of the chemical form was performed by HPLC at 15 and 60 min after injection as shown in Figure 4. HPLC analysis shows that approximately 80% of radioactivity was identified as *S*-[6-(7-methylpurinyl)]glutathione (7mPSG) (retention time of authentic sample, 15.3 min) both at 15 and 60 min post-injection. The peak height corresponding to 7m6CP (17.3 min) was very low at 15 min post-injection. Brain radioactivity principally existed in the form of [^{14}C]7mPSG during the period of 15–60 min after [^{14}C]7m6CP injection, although some unknown peaks slightly increased with time, which might be metabolites formed from the GSH conjugate by metabolic enzymes such as γ-glutamyltranspeptidase.[17] In addition to the efficiency of rapid conversion into the GSH conjugate, [^{14}C]7m6CP possessed specificity that means the production of the single probe within the brain.

The proprobe is essential for the delivery of radioactivity into the brain; however, it should be cleared from the blood as shown in Figure 1b. HPLC and TLC analyses of chemical form demonstrated that the amount of

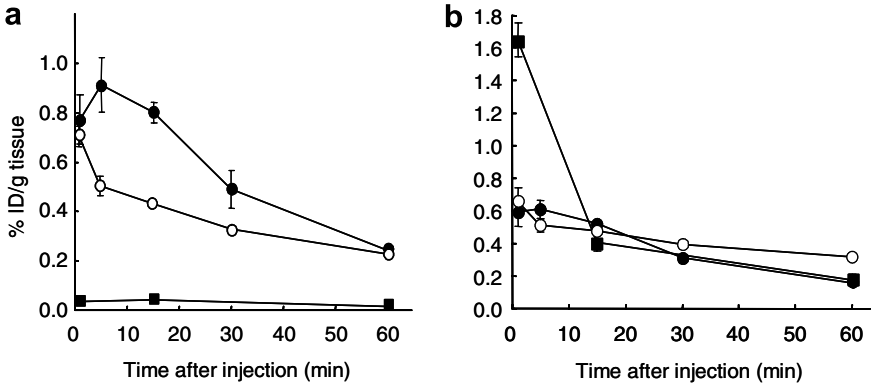

Figure 2. Time–radioactivity curves in the rat brain (a) and blood (b) after intravenous injection of [^{14}C]7m6CP (closed circles), [^{14}C]9m6CP (open circles), and [^{35}S]7mPSG (closed squares). Data are given as means ± the standard deviation ($n = 3$ rats for each point), and brain uptakes are corrected for the vascular volume of 3%, based on the radioactivity of the whole blood at each time-point. ID, injected dose.

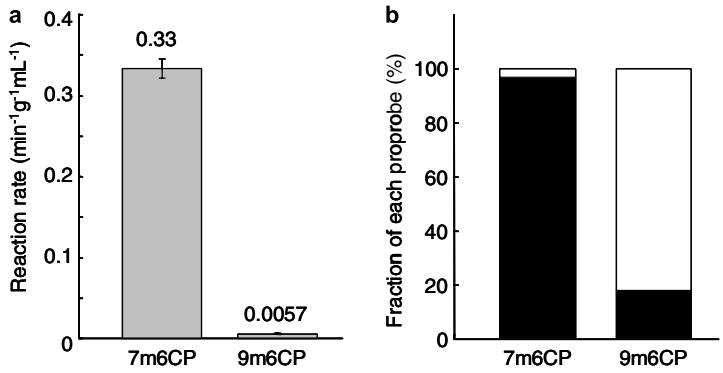

Figure 3. (a) The reaction rate of proprobes with GSH in rat brain homogenate. Data are given as means ± standard deviation. (b) Analysis of the chemical form in the rat brain by TLC at 15 min after the intravenous injection of [^{14}C]7m6CP (left) and [^{14}C]9m6CP (right). White and black areas denote the fraction of each proprobe and metabolite, respectively. Data are shown from a single experiment on each compound.

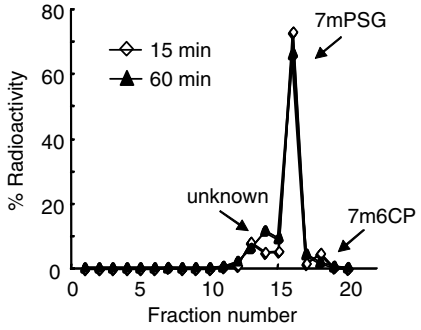

Figure 4. Analysis of the chemical form in rat brain. HPLC analysis shows that approximately 80% of radioactivity was identified as 7mPSG at 15 (diamond) and 60 min (triangle) following the administration of [^{14}C]7m6CP. The peak corresponding to 7m6CP was very low at 15 min. The retention times of authentic 7mPSG and 7m6CP are 15.3 and 17.3 min, respectively. Data are shown from a single experiment on each time-point.

[^{14}C]7m6CP in the brain was negligible from 15 min after the injection (Figs 3b and 4), implying that there is no additional delivery of [^{14}C]7m6CP into the brain from blood. This rapid clearance would be attributed to the metabolism by GSH/GST in the blood or peripheral tissues.

To confirm that 7mPSG cannot move into and out of the brain by simple diffusion, brain uptake was measured following the intravenous injection of [^{35}S]7mPSG into rats (Fig. 2a). The brain radioactivity of [^{35}S]7mPSG was much lower than that of the proprobe, [^{14}C]7m6CP, during the period of 1–60 min. This result verified that 7mPSG could not penetrate the BBB by simple diffusion. Nevertheless, it was eliminated from the brain, indicating that the carrier-mediated transport is involved in the extrusion.

Various transporters probably contribute to the 7mPSG efflux from the brain; however, GSH conjugates are well known to be good substrates for MRP1 that is involved in brain efflux systems.[4,5] The possibility for the involvement of MRP1 in the 7mPSG efflux was examined by a rapid filtration technique using membrane vesicles containing human MRP1 and MRP1-defective vesicles as controls. As shown in Figure 5, membrane vesicles containing MRP1 showed a significant time- and ATP-dependent uptake of [^{35}S]7mPSG; uptake of the compound into control vesicles was very low in both the absence and presence of ATP. This result suggested that MRP1 participated in the 7mPSG efflux from the brain. Although 7mPSG is a good substrate for MRP1, we cannot exclude possibilities that other efflux transporters such as MRP isoforms and P-glycoprotein contribute to the transport of 7mPSG. Moreover, the coordination of uptake transporters such as organic anion transporters and efflux transporters seems to be involved in the elimination of 7mPSG from the site within the brain to the blood, similar to the efflux transport of $E_2$17βG (a glucuronate conjugate).[9] Since MRP1 is also expressed at the choroid plexus,[3] 7mPSG is possibly extruded via the blood–cerebrospinal fluid barrier. Here, brain kinetics and chemical form analysis showed that the GSH conjugate (7mPSG) was extruded from the brain during the period of 15–60 min after the proprobe injection. Uptake values during that period were plotted in a semilog scale. As a result, the semilog plot exhibited linearity (multiple correlation coefficient: $R = 0.99$),

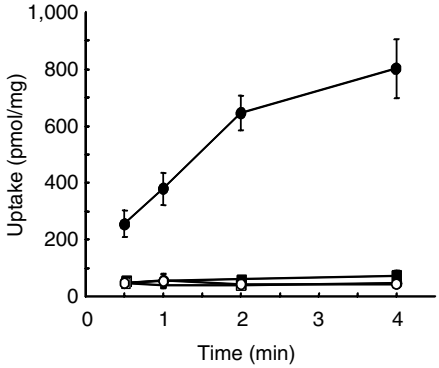

Figure 5. Transport of [^{35}S]7mPSG by MRP1. Membrane vesicles containing MRP1 (circles) or MRP1-defective vesicles (squares) were incubated at 37 °C with 50 μM [^{35}S]7mPSG in the presence of 4 mM ATP (closed symbols) or AMP (open symbols). Each point and bar is the means ± standard deviation of three experiments in duplicate.

indicating that 7mPSG was single-exponentially extruded from the brain. The first-order efflux rate constant (min^{-1}) of 7mPSG, estimated from the slope, was 0.025 ± 0.0013 (means ± standard error). Interestingly, this efflux rate estimated by MEM using Wistar rats is similar to the $E_2$17βG efflux rate ($0.028\ min^{-1}$) estimated by intracerebral injection using Sprague–Dawley rats,[9] implying that the same efflux systems might contribute to the 7mPSG efflux at the BBB. To investigate the involvement of efflux pathways and other transporters, further studies will be needed.

In conclusion, 7m6CP was newly designed as the proprobe to deliver its GSH conjugate, based on the concept of MEM. 7m6CP could successfully deliver the conjugate into the brain and appears useful for the noninvasive assessment of brain GSH-conjugate efflux systems.

3. Material and methods

3.1. General

All commercially available starting materials and solvents were of reagent grade or better and were used without further purification. [^{14}C]Methyl iodide (specific activity: 2.15 GBq/mmol) and [^{35}S]GSH (35.4 TBq/mmol) were purchased from Amersham Biosciences Corp. (Piscataway, NJ, USA) and PerkinElmer Life Sciences, Inc. (Boston, MA, USA), respectively. Thin layer chromatography (TLC) plates were purchased from Merck Ltd (Tokyo, Japan). Membrane vesicles containing human MRP1, or MRP1-defective vesicles as a control, were purchased from SOLVO Biotechnology, Inc. (Budapest, Hungary). Proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy was performed on a JEOL JNM-ALPHA 500 FT-NMR (JEOL Ltd, Tokyo, Japan) with chemical shifts reported in units of parts per million (ppm). Fast atom bombardment mass spectrometry (FAB-MS) was performed using a JEOL JMS-HX-110A mass spectrometer (JEOL Ltd, Tokyo, Japan). Radioactivity was measured using a liquid scintillation counter (LS 6000; Beckman Instruments, Fullerton, CA, USA), and the relative radioactivity of compounds on TLC was quantified using an imaging phosphor plates system (BAS 1800 system; Fuji Photo Film Co., Tokyo, Japan).

3.2. Synthesis

3.2.1. 6-Chloro-9-methylpurine (9m6CP) and 6-chloro-7-methylpurine (7m6CP). Methyl iodide (1.7 g) was added to a solution of 6-chloropurine (930 mg) and potassium carbonate (830 mg) dissolved in 30 mL of dimethylsulfoxide (DMSO). After the reaction mixture was stirred at room temperature for 24 h, DMSO was evaporated in vacuo, and then the residue was purified by column chromatography (silica gel C-200, ethyl acetate/ethanol, 9:1, v/v). In addition, products were recrystallized from benzene to give 9m6CP (35%, white solid) and 7m6CP (6.2%, light yellow solid). Each compound was identified by TLC and comparison of NMR data using an authentic sample purchased from Toronto Research Chemicals Inc. (North York, Ontario, Canada).

3.2.2. 6-Chloro-9-methylpurine. ^{1}H-NMR (δ, $CDCl_3$): 3.89 (3H, s, NCH_3), 8.06 (1H, s, H-8), 8.68 (1H, s, H-2); FAB-MS, *m*/*z*: 169 $(M+H)^+$. Found: 169; mp: 138–142 °C (lit.,[18] 143–144 °C).

3.2.3. 6-Chloro-7-methylpurine. ^{1}H-NMR (δ, DMSO-d_6): 4.07 (3H, s, NCH_3), 8.73 (1H, s, H-8), 8.77 (1H, s, H-2); FAB-MS, *m*/*z*: 169 $(M+H)^+$. Found: 169; mp: 196–197 °C (lit.,[19] 198–199 °C).

3.2.4. *S*-[6-(7-methylpurinyl)]glutathione (7mPSG). This compound was prepared by slight modification of the method as described previously.[20] 6-Chloro-7-methylpurine (26 mg) and GSH (105 mg) were dissolved in 760 μL each of 1 N NaOH solution and ethanol. After the mixture was heated for 4 h at 50 °C in a water bath, the solvent was evaporated, and ethanol was completely removed. Then, the concentrated residue was purified by column chromatography (Cosmosil 75 C_{18}–OPN, 1% aqueous AcOH) to give 7mPSG as a colorless solid (yield: 66%). ^{1}H-NMR (δ, D_2O): 1.93 (2H, m, Glu-γ-CH_2), 2.27 (2H, m, Glu-β-CH_2), 3.47 (1H, dd, $J = 8.1$, 14.2 Hz, Cys-β-CH_2), 3.54 (1H, d, $J = 17.1$ Hz, Gly-CH_2), 3.57 (1H, d, $J = 6.7$ Hz, Glu-α-CH_2), 3.61 (1H, d, $J = 17.1$ Hz, Gly-CH_2), 3.82 (1H, dd, $J = 4.6$, 14.2 Hz, Cys-β-CH_2), 3.87 (3H, s, NCH_3), 4.70 (1H, dd, $J = 4.6$, 8.1 Hz, Cys-α-CH_2), 8.09 (1H, s, H-8), 8.46 (1H, s, H-2); FAB-MS, *m*/*z*: 440 $(M+H)^+$. Found: 440; mp: 157–165 °C (dec).

3.2.5. Radiochemical synthesis. 6-Chloro-9-[^{14}C]methylpurine ([^{14}C]9m6CP) and 6-chloro-7-[^{14}C]methylpurine ([^{14}C]7m6CP) were prepared by N-methylation using [^{14}C]methyl iodide. To a mixture of 6-chloropurine (9.3 mg) and potassium carbonate (9.7 mg), a solution of 9.25 MBq of [^{14}C]methyl iodide in 1 mL of acetone was added. In a sealed vial, the mixture was heated in a water bath at 55 °C for approximately 30 min, and then acetone was removed in a stream of nitrogen. The residue was dissolved in 200 μL acetone and applied to TLC with a silica-gel plate. The developing solvents, [^{14}C]9m6CP and [^{14}C]7m6CP, were a mixture of chloroform/ethanol (9:1; v/v) and ethyl acetate/ethanol (9:1; v/v), respectively. The radioactive zone with an R_f value corresponding to the authentic compound was scratched, collected, and extracted with acetone. Radiochemical yields of [^{14}C]9m6CP and [^{14}C]7m6CP were 50% and 10–20%, respectively. Radiochemical purities (99%) were determined by TLC using the solvent systems as described above.

3.2.6. [^{35}S]*S*-[6-(7-methylpurinyl)]glutathione ([^{35}S]7mPSG). [^{35}S]7mPSG was synthesized enzymatically by the conjugation of 7m6CP with [^{35}S]GSH using glutathione *S*-transferase (GST) from equine liver (purchased from Sigma). [^{35}S]GSH (3.7 MBq) was dissolved in 150 μL of phosphate buffer (0.1 M, pH 7.4), and the solution was extracted with ethyl acetate to remove excess dithiothreitol. A mixture of 7m6CP (3 mg) and GST (0.3 mg) dissolved in 100 μL of the phosphate buffer was added to the solution of

[^{35}S]GSH, and the solution was incubated at 37 °C for 1 h. After incubation, the solution was concentrated to a final volume of ca. 100 μL under a stream of nitrogen, and the solution to which 50 μL of ethanol was further added was applied to a TLC plate (RP-18 F_{254s}). The TLC plate was developed using water/acetonitrile/AcOH (9:1:0.1, v/v/v) as an eluent. After visualization by autoradiography, [^{35}S]7mPSG was scraped from the plate and extracted from C18 with 50% ethanol in water. The extracted solution was filtered through a 0.45 μm filter, and analyzed by HPLC under the conditions described below. Radiochemical yield was 50%, and radiochemical purity (97%) was calculated from the radioactivity in the eluate fraction corresponding to the authentic sample and the total radioactivity injected into HPLC.

Since these compounds ([^{14}C]7m6CP, [^{14}C]9m6CP, and [^{35}S]7mPSG) were prepared without adding carriers, each specific activity may be considered to be equal to that of the original radioactive compound, $^{14}CH_3I$ or [^{35}S]GSH.

3.2.7. Animal studies. Wistar rats weighing 185–205 g (male, 8 weeks old) were purchased from Japan SLC Inc. (Shizuoka, Japan). In the present study, they were treated and handled according to the 'Recommendations for Handling of Laboratory Animals for Biomedical Research', compiled by the Committee on Safety and Ethical Handling Regulations for Laboratory Animal Experiment, NIRS.

3.2.8. Reaction rates of purine derivatives with GSH in tissue homogenate. Rats were anesthetized with ether and decapitated. The cortex was removed and homogenized in phosphate buffer (0.1 M, pH 7.4). The homogenate (250 μL) supplemented with 2 mM GSH was placed in tubes and preincubated at 37 °C for 30 min. Each [^{14}C]-purine solution (111 kBq in 20 μL buffer) was added to each tube to initiate the reaction. At designated intervals, 25 μL of the homogenate was immediately added to 50 μL of the stop solution, ethanol containing excess diethyl maleate (ca. 50 mM), and then centrifuged. The supernatant (5 μL) was applied to TLC with a silica-gel plate, and developed with ethyl acetate/ethanol (9:1; v/v). Then, the air-dried TLC plate was covered with a 5 μm thick lumilar membrane and placed in a cassette in contact with an imaging phosphor plate for several hours. Radioactivity corresponding to the R_f value of the unchanged purines was quantified using BAS. The first-order rate constants were calculated by plotting the change in the log of the radioactivity of [^{14}C]-purine derivatives against time, and corrected for the concentration of the homogenate to obtain the reaction rates ($min^{-1}\,g^{-1}\,mL^{-1}$). All determinations were made in triplicate.

3.2.9. Time–radioactivity curves. Each labeled compound dissolved in saline (111 kBq/200 μL) was injected via a lateral tail vein. Rats, anesthetized with ether immediately before decapitation, were decapitated at 1, 5, 15, 30, and 60 min after the injection ($n = 3$ per time). Blood samples were collected and weighed. Protosol (1 mL) (Packard Bioscience, Groningen, The Netherlands) was added to blood samples, and then hydrogen peroxide was added for bleaching. Cerebral cortexes were dissected, weighed, and then Soluene-350 (Packard Bioscience) was added to dissolve the tissue at 60 °C, after which radioactivity was measured using a liquid scintillation counter. The tissue uptake of ^{14}C or ^{35}S activity was expressed as the percentage of injected dose per gram tissue (% ID/g tissue).

3.2.10. Analysis of the chemical form in rat brain. For TLC analysis, rats were decapitated 15 min after [^{14}C]9m6CP or [^{14}C]7m6CP (ca. 2.2 MBq) injection. The head was quickly dipped in liquid nitrogen and the brain was removed. The brain was homogenized in 50% ethanol in water and then centrifuged. The supernatant was applied to a silica-gel TLC plate and developed using ethyl acetate/ethanol (9:1; v/v). Then, the radioactivity on TLC was quantified using BAS. For HPLC analysis, rats were decapitated at 15 and 60 min after [^{14}C]7m6CP injection. The brain was treated and homogenized as described above, and one half-volume of ethanol was added to the homogenate to be centrifuged. The supernatant was concentrated in a stream of nitrogen, followed by centrifugation and filtration through a 0.45 μm filter. The concentrated sample was analyzed by HPLC under the conditions described below. Both the recovery of radioactivity in HPLC elutions and the extraction efficiency of that to the supernatant were more than 95%.

3.2.11. Vesicular transport assay. Transport studies were performed using the rapid filtration technique based on the assay protocol of SOLVO Biotechnology, Inc. with a slight modification. Briefly, 73.5 μL of transport medium [40 mM MOPS–Tris, 50 mM KCl, 6 mM $MgCl_2$, 10 μL of membrane vesicle suspension (50 μg of protein), [^{35}S]7mPSG (3.7 kBq), 50 μM 7mPSG, and 2 mM GSH] was preincubated at 37 °C for 5 min, and then a transport assay was initiated by the addition of 1.5 μL of Mg–ATP (0.2 M) or Mg–AMP. The transport reaction was stopped by the addition of 1 mL ice-cold buffer containing 40 mM MOPS–Tris and 70 mM KCl. The stopped reaction mixture was filtered through a GF/F glass fiber filter (Whatman, Maidstone, England) and then washed twice with 5 mL of the ice-cold buffer. Radioactivity retained on the filters was determined using a liquid scintillation counter.

3.2.12. Estimation of efflux rate constant (k_{eff}). Rats were decapitated 15, 30, and 60 min after the intravenous injection of [^{14}C]7m6CP, and then cerebral cortexes were dissected and weighed. The radioactivity in the tissue was measured as described above. Nine data corrected for vascular volume ($n = 3$, each point) were fitted to the exponential function by the least-square method to obtain the efflux rate constant (min^{-1}).

3.2.13. HPLC analysis. Unlabeled compounds were analyzed using an HPLC system consisting of a model L-7100 pump (Hitachi, Tokyo, Japan), a model L-7450/L-7400 UV absorbance detector set at 288 nm (Hitachi, Tokyo, Japan), and a cosmosil 5C18-AR-II column (4.6 I.D. × 250 mm) (Nacalai Tesque, Kyoto,

Japan). The column was eluted with mobile phase (acetonitrile and water containing 0.1% trifluoroacetic acid) at a flow rate of 1.0 mL/min. The acetonitrile gradient was changed from 0% to 10% over 10 min, kept constant at 10% for 5 min, and increased to 20% over 5 min. [^{35}S]7mPSG and brain chemical form were analyzed under the same HPLC systems, and eluate from the column was collected in 1 mL fractions. Radioactivity in the fractions was measured by a liquid scintillation counter.

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