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Reactivity of 6-Halopurine Analogs with Glutathione as a Radiotracer for Assessing Function of Multidrug Resistance-Associated Protein 1

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6-Bromo-7-[^{11}C]methylpurine is reported to react with glutathione via glutathione S-transferases in the brain and to be converted into a substrate for multidrug resistance-associated protein 1 (MRP1), an efflux pump. The compound with a rapid conversion rate allows quantitative assessment of MRP1 function, but this rate is probably susceptible to interspecies differences. Hence, for application to different species, including humans, it is necessary to adjust the conversion rate by modifying the chemical structure. We therefore designed 6-halo-9-(or 7)-[^{14}C]methylpurine (halogen: F, Cl, Br, or I), and evaluated them in vitro with respect to enzymatic reactivity with glutathione using brain homogenates from the mouse, rat, or monkey. There was a marked difference in reactivity between these species. Changes in the position of the methyl group and halogen on *N*-methyl-6-halopurine provided various compounds possessing wide-ranging reactivity with glutathione. In conclusion, the adjustment of reactivity of 6-bromo-7-[^{11}C]methylpurine may allow assessment of MRP1 function in the brain in various species.

Introduction

Multidrug resistance-associated protein 1 (MRP1[a]), a member of the ATP binding cassette superfamily of transporters, contributes to various physiological functions, including defense against xenobiotics and toxic metabolites, as well as protection from the toxic effect of oxidative stress.[1−4] This protein has an unusually broad substrate specificity and can transport numerous hydrophobic compounds, including therapeutic drugs and hydrophilic compounds.[5] It has been reported that adriamycin, a chemotherapeutic agent, affects MRP1 expression in the mouse brain.[6] A slight increase in MRP1 expression was observed in hippocampal samples obtained from patients with Alzheimer's disease.[7] Therefore, an in vivo quantitative assessment of MRP1 function in the brain is useful for examining the alteration of MRP1 expression caused by brain diseases and for directly evaluating the effect of drugs, including modulators used to improve drug delivery to the brain, on MRP1.

6-Bromo-7-[^{11}C]methylpurine has recently been developed as a radiotracer for quantitative assessment of MRP1 function in the living brain using positron emission tomography (PET).[8] This radiotracer was designed based on the metabolite extrusion method (MEM), a type of prodrug/drug approach.[8−10] Figure 1 shows the rationale of MEM for assessing MRP1 function in the brain. 6-Bromo-7-[^{11}C]methylpurine in the blood crosses the blood−brain barrier after intravenous injection. It is immediately converted to a hydrophilic substrate for MRP1, a glutathione (GSH) conjugate, in the brain. This allows assessment of MRP1 function by an estimation of the efflux rate of the substrate from the time−radioactivity curve after complete disappearance of the unconjugated radiotracer. The efflux rate of the GSH conjugate *S*-[6-(7-methylpurinyl)]glutathione (PSG) was estimated with high precision and decreased by approximately 90% in Mrp1 knockout mice compared with wild-type mice.[8]

Small-animal models of human disease, in particular, mouse and rat models, are valuable for investigating the underlying mechanisms of human disease. Nonhuman primate studies provide a crucial link between small-animal and human studies. It is therefore desirable to assess MRP1 function in various species. In the MEM, the conversion rate is an extremely important factor because a radiotracer with a low conversion rate in the brain does not allow quantitative assessment of MRP1 function in the brain. GSH conjugation is typically mediated by GSH S-transferases (GSTs), and thus the conversion rate is probably susceptible to interspecies differences.[11,12] It is therefore necessary to adjust the conversion rate by modifying the chemical structure of 6-bromo-7-[^{11}C]methylpurine for application of the MEM to different types of species, including humans. This requires examination of the effect of substituents of this compound on the conversion rate.

We designed and synthesized 6-halo-9-[^{14}C]methylpurines (9-isomer) and 6-halo-7-[^{14}C]methylpurines (7-isomer) based on 6-bromo-7-[^{11}C]methylpurine (Table 1). We examined nonenzymatic and enzymatic reaction rates with GSH in brain homogenates from the mouse, rat, or monkey under tracer dose conditions. The lowest unoccupied molecular

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[a] Abbreviations: S_NAr, aromatic nucleophilic substitution; GSH, glutathione; GSTs, glutathione *S*-transferases; LUMO, lowest unoccupied molecular orbital; MEM, metabolite extrusion method; MRP1, multidrug resistance-associated protein 1; PET, positron emission tomography; PSG, *S*-[6-(7-methylpurinyl)]glutathione.

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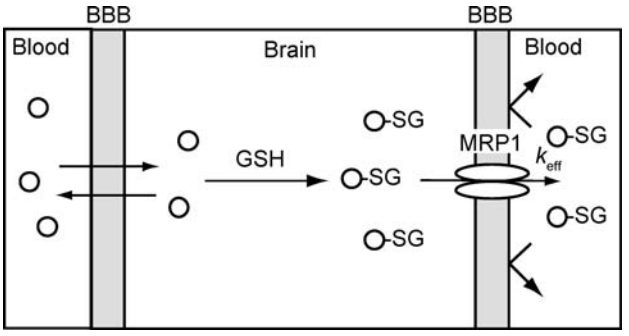
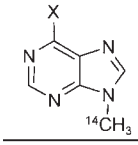
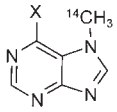


Figure 1. MEM for assessment of MRP1 function in the brain. After a radiotracer (open circles) penetrates the blood−brain barrier (BBB) by diffusion, it is attacked by the nucleophilic thiolate anion of GSH to form its GSH conjugate (circle-SG), which is extruded by MRP1 from the brain to the blood. The efflux rate constant (k_{eff}) is influenced by the pumping action of MRP1. Brain radioactivity is constituted by a mixture of the radiotracer and conjugate in the early phase after injection but only the conjugate after complete conversion. The conversion rate is therefore important for quantitative assessment of MRP1 function in the living brain.

Table 1. Analogs of 6-Halopurine

Structure	Compound	X
	**1a**	F
	1b	Cl
	1c	Br
	1d	I
	**2a**	F
	2b	Cl
	2c	Br
	2d	I

orbital (LUMO) energy values were determined to describe the electrophilicity of purine analogs.

Results

Chemistry. Radiotracers were synthesized by methylation of 6-halopurine analogs with $^{14}CH_3I$ as a model for $^{11}CH_3I$ in acetone. This resulted in the formation of two isomers: the major product being the 9-isomer (**1a**−**1d**) and the minor product being the 7-isomer (**2a**−**2d**). Their radiochemical purities were >95%. The radiochemical yield, although not optimized, was 35−50% for the 9-isomer and 8−20% for the 7-isomer, which was similar to an earlier study on alkylation of 6-chloropurine.[13] The low yield of the 7-isomer was probably due to the steric hindrance between the halogen at the 6-position and the methyl group at the 7-position. The radiotracers were rapidly labeled with ^{14}C by a one-step reaction. They were therefore expected to be well suited for labeling with ^{11}C nuclide for PET studies after slight modification. Indeed, 6-bromo-7-[^{11}C]methylpurine was obtained in a total synthesis time of 30 min, including HPLC purification and formulation.[8]

Nonenzymatic Reactivity. Because a small amount of a radiotracer is injected into humans or animals in PET studies, reactivity of 6-halopurine analogs with GSH was investigated under tracer dose conditions. Table 2 shows the nonenzymatic reaction rate (k_{non}) of ^{14}C-labeled 6-halopurine analogs with GSH (physiological concentration, 2 mM)[14] in phosphate buffer. The rank order of k_{non} was **1a** > **1d** ≈ **1c** > **1b** for the 9-isomer and **2a** > **2d** ≥ **2c** > **2b** for the 7-isomer. This was similar to the rank order for aromatic nucleophilic substitution (S_NAr) reactions of 6-halopurine nucleosides with thiolate nucleophiles (thioacetate and 3-methylbutane-1-thiol) in organic solvents: F > Br ≈ I > Cl.[15] Most S_NAr reactions are thought to proceed via a Meisenheimer complex or a σ-complex intermediate.[16] In S_NAr reactions, the relative reactivity of the halogen is dependent upon the rate-limiting step, which is formation or decomposition of the σ-complex intermediate. In a reaction of 1-substituted-2,4-dinitrobenzenes with piperidine, the rank order was F ≫ Br ≈ Cl > I.[17] The differences in rate between Cl, Br, and I were small, and the maximum variation was only 4-fold. When the 1-substituent was F, the relative rate was 3300 compared with I. This behavior would not be expected in a reaction in which decomposition of the σ-complex is rate limiting. Given these findings, our result suggested that σ-complex formation was the rate-limiting step for the nonenzymatic reaction of 6-halopurine analogs with GSH.

Table 2. Nonenzymatic Reactivity with GSH

cmpd	position[a]	X	k_{non}[b] (h^{-1})
1a	9	F	0.41 (0.013)
1b	9	Cl	0.024 (0.0060)
1c	9	Br	0.046 (0.0036)
1d	9	I	0.046 (0.0036)
2a	7	F	1.4 (0.036)
2b	7	Cl	0.096 (0.012)
2c	7	Br	0.28 (0.0060)
2d	7	I	0.33 (0.012)

[a] Position of the methyl group on the purine ring. [b] Rate constant in phosphate buffer (0.1 M, pH 7.4) containing 2 mM GSH. Values are the mean of triplicate determinations with standard deviation in parentheses.

Comparing the effect of the methyl position (9 or 7), the k_{non} of the 7-isomer was several times higher than that of the 9-isomer, despite the fact that the methyl group at the 7-position was supposed to sterically hinder the thiolate anion of GSH from approaching the purine carbon at the 6-position. Introduction of a methyl group into the 7-position of the purine appeared to enhance the electrophilicity of 6-halopurine analogs, which was supported by the difference in LUMO energy between the 9-isomer and the 7-isomer, although the LUMO energy did not reflect the high reactivity of fluorine (Table 3).

Enzymatic Reactivity. Table 4 shows the enzymatic reaction rate (k_{enz}) of 6-halopurine analogs in brain homogenates from the mouse, rat, and monkey supplemented with 2 mM GSH, and Table 5 exhibits the ratio of k_{enz} to k_{non} for each compound. There was a marked difference in k_{enz} between species, and the rank order of k_{enz} among compounds was not identical. As expected, k_{enz} of each 6-halopurine analog was much higher than k_{non}. Considering that **2b** and **2c** are rapidly converted into a GSH conjugate of PSG in the rodent brain in vivo,[8,10] these results indicate that GSTs would catalyze the reaction of 6-halopurine analogs with GSH. GSTs have two substrate-binding sites: the GSH-binding site and the substrate-binding site for hydrophobic compounds.[18] The partition coefficient of 6-halopurine analogs was determined to investigate the relationship between

Table 3. Comparison of Electrophilic Characteristics between the 9-Isomer and the 7-Isomer

			E(LUMO) (eV)[b]	
cmpd	position[a]	X	AM1	PM3
1a	9	F	−0.784	−0.900
1b	9	Cl	−0.763	−0.830
1c	9	Br	−0.838	−0.865
1d	9	I	−0.831	−0.767
2a	7	F	−0.806	−0.960
2b	7	Cl	−0.793	−0.913
2c	7	Br	−0.859	−0.923
2d	7	I	−0.848	−1.09

[a] Position of the methyl group on the purine ring. [b] LUMO energy was calculated with the AM1 and PM3 Hamiltonian.

Table 4. Enzymatic Reactivity with GSH

			k_{enz}[b] (h^{-1} g^{-1} mL^{-1})		
cmpd	position[a]	X	mouse	rat	monkey
1a	9	F	8.4 (0.091)	4.2 (0.084)	32 (0.19)
1b	9	Cl	1.5 (0.14)	0.43 (0.0090)	2.0 (0)[c]
1c	9	Br	3.6 (0.26)	1.5 (0.069)	2.0 (0.019)
1d	9	I	2.6 (0.079)	2.0 (0.035)	1.3 (0.070)
2a	7	F	41 (0.9)	47 (3.9)	120 (4.5)
2b	7	Cl	21 (0.68)	31 (0.73)	7.8 (0.033)
2c	7	Br	83 (1.2)	92 (4.6)	26 (0.067)
2d	7	I	150 (3.4)	260 (14)	80 (0.51)

[a] Position of the methyl group on the purine ring. [b] Rate constant in cerebral cortical homogenate from three species supplemented with 2 mM GSH. Values are the mean (n = 3, rat) and the mean of triplicate determinations of homogenate from a single monkey or the combined homogenate from seven mice. Standard deviation is shown in parentheses. [c] All values are identical.

Table 5. Ratio of Enzymatic (k_{enz}) to Nonenzymatic (k_{non}) Reaction Rates of Each Compound

			k_{enz}/k_{non}		
cmpd	position[a]	X	mouse	rat	monkey
1a	9	F	21	10	79
1b	9	Cl	64	18	84
1c	9	Br	78	32	42
1d	9	I	57	44	29
2a	7	F	28	33	85
2b	7	Cl	214	321	81
2c	7	Br	295	325	93
2d	7	I	462	781	241

[a] Position of the methyl group on the purine ring.

k_{enz} and hydrophobicity (Figure 2). The partition coefficients of the 7-isomer were approximately half of those of the corresponding 9-isomer, and the rank order of the partition coefficient for each isomer was I > Br > Cl > F. Partition coefficients were approximately correlated with the ratio of k_{enz}/k_{non} of the 7-isomer for each species and of the 9-isomer for the rat, suggesting that hydrophobicity in addition to nonenzymatic reactivity may contribute to the degree of k_{enz}.

Discussion and Conclusions

For the application of the MEM to different kinds of species, including humans, we investigated k_{enz} and k_{non} of 6-halopurine analogs, which can be used to estimate the in vivo conversion rate in the MEM. The nonenzymatic reactivity presumably contributes to the nonspecific reaction of

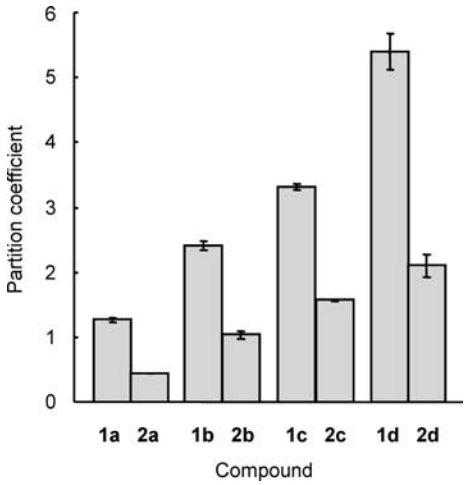


Figure 2. Hydrophobic nature of 6-halopurine analogs. Partition coefficients were determined in a mixture of 1-octanol/phosphate buffer (0.1 M, pH 7.4). Data are mean ± standard deviation of triplicate determinations.

6-halopurine analogs with other thiol groups (e.g., proteins and cysteine). Because 6-halopurine analogs possess high k_{enz}/k_{non}, they specifically produce an MRP1 substrate of the GSH conjugate. Similarly to **2c**, **2b** was efficiently converted to the GSH conjugate of PSG in the rat brain in vivo,[8,10] although k_{enz} of **2b** was lower than that of **2c**. Brain radioactivity was almost completely constituted by PSG from 15 min after intravenous injection of **2b** or **2c**. Radiotracers with greater than the rat k_{enz} of **2b** (31 h^{-1} g^{-1} mL^{-1}) would therefore satisfy the requirement of an efficient conversion in the MEM (Table 4). The 7-isomer appeared to be almost applicable to the mouse, rat, and mokey, but the 9-isomer (except for **1a**) showed low reactivity with GSH in these species. The low reactivity of the 9-isomer would delay complete disappearance of the unchanged form in the brain in vivo. This may make estimation of the efflux rate of the GSH conjugate inaccurate. However, if sufficient brain radioactivity persists for a long time, the extension of duration of PET may allow accurate estimation of the efflux rate. The 9-isomer is therefore not always a poor radiotracer for assessment of MRP1 function.

In addition to reactivity with GSH, the MRP1-mediated transport rate may be susceptible to interspecies differences. We examined the kinetics of human MRP1- and rat Mrp1-mediated transport of PSG, the GSH conjugate of the promising radiotracer (the 7-isomer). The K_m (Michaelis−Menten constant) and V_{max} (maximum uptake rate) for human MRP1 were 127 ± 18 μM and 1051 ± 55 pmol/min/mg protein, respectively, whereas those for rat Mrp1 were 78.9 ± 10 μM and 1053 ± 42 pmol/min/mg protein, respectively. Assuming that the expression level of MRP1 in human brain is equal to that of Mrp1 in rat brain, there may not be a critical difference between the efflux rate in humans and rats, suggesting that the ^{11}C-labeled 7-isomer may be applicable to human studies.

In conclusion, reactivity of **2c** with GSH can be adjusted by changing the position of the halogen and the methyl group. This may allow assessment of MRP1 function in the brain in different species, including humans.

Experimental Section

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 (36.1 TBq/mmol) were purchased from Amersham Biosciences Corp. (Piscataway, NJ) and PerkinElmer Life Sciences, Inc. (Boston, MA), respectively. Thin-layer chromatography (TLC) plates were purchased from Merck Ltd. (Tokyo, Japan). Membrane vesicles containing human MRP1 or rat Mrp1 were purchased from BD Biosciences (Tokyo, Japan). Proton nuclear magnetic resonance (^{1}H NMR) spectroscopy was carried out on 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) and electron impact mass spectrometry (EI-MS) were carried out using a JEOL JMS-HX-110A mass spectrometer and a GC-MATE mass spectrometer (JEOL Ltd.). Radioactivity was measured using a liquid scintillation counter (LS 6000; Beckman Instruments, Fullerton, CA). The relative radioactivity of compounds on TLC was quantified using an imaging phosphor plate system (BAS 1800 system; Fuji Photo Film Co., Tokyo, Japan). Purity of the test compounds was determined by TLC or HPLC to be greater than 95%.

6-Fluoropurine. This compound was prepared by nucleophilic displacement of the trimethylammonio group with potassium hydrogen fluoride, after the synthesis of trimethylpurin-6-ylammonium chloride from 6-chloropurine, as previously described.[19] ^{1}H NMR (δ, CD_3OD) 8.50 (1H, s, H-8), 8.59 (1H, s, H-2); FAB-MS (m/z) calcd, 139 $(M + H)^+$; found, 139; yield 17%.

6-Iodopurine. This compound was prepared by reaction of 6-chloropurine with hydriodic acid, as previously described.[20] ^{1}H NMR (δ, DMSO-d_6) 8.58 (1H, s, H-8), 8.63 (1H, s, H-2), 13.8 (1H, br, NH); FAB-MS (m/z) calcd, 247 $(M + H)^+$; found, 247; yield 72%.

Nonradiolabeled 6-Halo-9-methylpurine and 6-Halo-7-methylpurine. These compounds were prepared by a standard alkylation procedure as previously reported.[13,19] Briefly, methyl iodide (12 mmol) was added to a solution of 6-halopurine (6 mmol) and potassium carbonate (6 mmol) dissolved in 30 mL of dimethylsulfoxide. After the reaction mixture was stirred at room temperature for 24 h, the solvent was evaporated in vacuo. The residue was then purified by column chromatography (silica gel C-200) using one of the following eluent systems: (A) ethyl acetate/ethanol (9:1), (B) chloroform/ethanol (9:1), (C) ethyl acetate/ethanol (95:5), and (D) ethyl acetate.

6-Fluoro-9-methylpurine. ^{1}H NMR (δ, $CDCl_3$) 3.92 (3H, s, NCH_3), 8.06 (1H, s, H-8), 8.59 (1H, s, H-2); EI-MS (m/z) calcd, 152 (M^+); found, 152; eluent system (A); yield 36%; mp 130−132 °C (lit.,[21] 135−136 °C).

6-Fluoro-7-methylpurine. ^{1}H NMR (δ, $CDCl_3$) 4.07 (3H, s, NCH_3), 8.16 (1H, s, H-8), 8.72 (1H, s, H-2); EI-MS (m/z) calcd, 152 (M^+); found, 152; eluent system (B); yield 13%; mp 140−142 °C.

6-Chloro-9-methylpurine and 6-Chloro-7-methylpurine. These compounds were characterized as previously reported.[10]

6-Bromo-9-methylpurine. This compound was recrystallized from benzene after separation by column chromatography. ^{1}H NMR (δ, $CDCl_3$) 3.92 (3H, s, NCH_3), 8.10 (1H, s, H-8), 8.71 (1H, s, H-2); EI-MS (m/z) calcd, 212 and 214 (M^+); found, 212 and 214; eluent system (C); yield 28%; mp 157−159 °C.

6-Bromo-7-methylpurine. This compound was characterized as reported.[8]

6-Iodo-9-methylpurine. This compound was recrystallized from chloroform after separation by column chromatography. ^{1}H NMR (δ, DMSO-d_6) 3.83 (3H, s, NCH_3), 8.61 (1H, s, H-8), 8.63 (1H, s, H-2); FAB-MS (m/z) calcd, 261 $(M + H)^+$; found, 261; eluent system (D); yield 20%; mp >211 °C dec (lit.,[22] 212−215 °C dec.)

6-Iodo-7-methylpurine. This compound was recrystallized from chloroform after separation by column chromatography. ^{1}H NMR (δ, DMSO-d_6) 4.07 (3H, s, NCH_3), 8.59 (1H, s, H-8), 8.72 (1H, s, H-2); FAB-MS (m/z) calcd, 261 $(M + H)^+$; found, 261; eluent system (D); yield 1.0%; mp >190 °C dec.

***S*-[6-(7-Methylpurinyl)]glutathione.** This compound was prepared from 6-chloro-7-methylpurine with GSH in the same manner as that described previously[10] and was analyzed using an HPLC system consisting of a model L-2130 pump (Hitachi High-Technologies Corporation, Tokyo, Japan), a model L-2420 UV−vis absorbance detector set at 288 nm (Hitachi High-Technologies Corporation), and a COSMOSIL 5C18-AR-II column (4.6 I.D. × 250 mm; Nacalai Tesque Inc.). The column was eluted with a mobile phase of acetonitrile/water containing 0.1% trifluoroacetic acid (10:90; v/v) at a flow rate of 1.0 mL/min.

Radiochemical Synthesis. 6-Halo-9-[^{14}C]methylpurines (9-isomer, **1a**−**1d**) and 6-halo-7-[^{14}C]methylpurines (7-isomer, **2a**−**2d**) were prepared by *N*-methylation using [^{14}C]methyl iodide. A solution of 9.25 MBq of [^{14}C]methyl iodide in 1 mL of acetone was added to a mixture of 6-halopurine (60 μmol) and potassium carbonate (70 μmol). In a sealed vial, the mixture was heated in a water bath at 55 °C for 30 min. Acetone was removed in a stream of nitrogen. The residue was dissolved in 200 μL of acetone and applied to silica gel TLC plates. It was developed with a mixture of chloroform/ethanol (9:1) for the 9-isomer or ethyl acetate/ethanol (9:1) for the 7-isomer. The radioactive zone with an R_f value corresponding to the authentic compound was scratched, collected, and extracted with acetone. Radiochemical yields of the 9-isomer and the 7-isomer were 35−50% and 8−20%, respectively. Radiochemical purities (>95%) were determined by TLC using chloroform/ethanol (9:1; v/v) and ethyl acetate/ethanol (9:1; v/v).

[^{35}S]*S*-[6-(7-methylpurinyl)]glutathione was synthesized by the conjugation of 6-chloro-7-methylpurine with [^{35}S]GSH. A solution of 6-chloro-7-methylpurine (2.2 mg) dissolved in 200 μL of ethanol was added to a solution of 9.25 MBq of [^{35}S]GSH in 225 μL of phosphate buffer (0.1 M, pH 7.4). In a sealed vial, the mixture was heated in a water bath at 55 °C for 60 min, and the solution was concentrated in a stream of nitrogen. The residue was purified by preparative TLC, as reported previously,[10] and was analyzed using the HPLC system as described above. The eluate from the column was collected in 0.5 mL fractions, and radioactivity in the fractions was measured by a liquid scintillation counter. Radiochemical yield was 60%; the radiochemical purity (98%) was calculated from the radioactivity in the eluate fraction corresponding to the authentic sample and the total radioactivity injected into HPLC.

These compounds were prepared without adding carriers, thus, each specific activity may be considered to be equal to that of the original radioactive compound, $^{14}CH_3I$ or [^{35}S]GSH.

Reaction Rates of Purine Analogs with GSH in Phosphate Buffer. A solution (2 mM GSH in 0.1 M phosphate buffer, pH 7.4; 150 μL) was placed in tubes and preincubated at 37 °C for 15 min. Addition of each [^{14}C]-purine solution (18.5 kBq in 10 μL buffer) to each tube initiated the reaction. At designated intervals, 20 μL of the solution was immediately added to 40 μL of ethanol containing excess diethyl maleate (50 mM), the latter acting as a stop solution. The resultant solution (5 μL) was applied to silica gel TLC plates, and developed with ethyl acetate/ethanol (9:1; v/v). The air-dried TLC plate was covered with a thin plastic film (lumilar film) and placed in a cassette in contact with an imaging phosphor plate for several hours. Radioactivity corresponding to the R_f value of unchanged purines was quantified using the BAS 1800 system. The pseudo first-order rate constants (k_{non}) were calculated by plotting changes in the log of the radioactivity of [^{14}C]-purine analogs against time. Determinations were made in triplicate.

Reaction Rates of Purine Analogs with GSH in Tissue Homogenates. Animals 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.

Male ddY mice (35−38 g; 8 weeks old) and Wistar rats (185−205 g; 8 weeks old) were purchased from Japan SLC

Inc. (Shizuoka, Japan). Nonanesthetized mice and rats anesthetized with diethyl ether were decapitated. Their brains were removed and the cerebral cortices dissected. A rhesus monkey (23 years old) was anesthetized with pentobarbital and killed by saline perfusion. The monkey brain was removed and stored at −80 °C before use; the tissue of the mouse and rat was used immediately after dissection. The cerebral cortex was weighed and homogenized in phosphate buffer (0.1 M, pH 7.4). The homogenate (150 μL) supplemented with 2 mM GSH was placed in tubes and preincubated at 37 °C for 15 min. Each [^{14}C]-purine solution (18.5 kBq in 10 μL buffer) was added to each tube to initiate the reaction. At designated intervals, 20 μL of the homogenate was immediately added to 40 μL of the stop solution described above and then centrifuged. The supernatant (5 μL) was applied to silica gel TLC plates, and developed with ethyl acetate/ethanol (9:1; v/v). First-order rate constants were calculated as described above and corrected for homogenate concentration after subtracting k_{non} to yield the reaction rate k_{enz} (h^{-1} g^{-1} mL^{-1}). Experiments were carried out with rat homogenate ($n = 3$) and in triplicate with homogenate from a single monkey or the combined homogenate from seven mice.

Partition Coefficient. Each ^{14}C-labeled purine analog was added to a 1:1 mixture of 1-octanol and 0.1 M phosphate buffer (pH 7.4), shaken vigorously, and allowed to equilibrate for 1 h at room temperature. ^{14}C concentrations in the organic and aqueous phases were measured with a liquid scintillation counter. Each partition coefficient was calculated as the ratio of concentration in the organic phase to that in the aqueous phase. Determinations were made in triplicate.

Molecular Orbital Calculations. Molecular orbital calculations were carried out on computers based on Intel Pentium 4 running on Windows XP. The semiempirical molecular orbital method was used, applying the AMI or PM3 Hamiltonian from the MOPAC program. Calculations were carried out using PRECISE criteria. Molecular structures were optimized using eigenvector Following (a geometry optimization procedure within MOPAC2002).

Vesicular Transport Studies. Transport studies were carried out using the rapid filtration technique[10,23] with some modification. Briefly, membrane vesicles (50 μg of protein) were incubated in the presence of 4 mM ATP or AMP in 50 μL of transport medium (40 mM MOPS-Tris, 50 mM KCl, 6 mM $MgCl_2$, 5.2 kBq of [^{35}S]PSG with 5, 15, 50, 150, or 500 μM of unlabeled PSG, 2 mM GSH) at 37 °C. The transport reaction was stopped at a given time by the addition of 200 μL of 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) and then washed five times with 200 μL of the ice-cold buffer. Radioactivity retained on the filters was determined using a liquid scintillation counter. ATP-dependent transport was calculated by subtracting the activity values obtained with AMP from those in the presence of ATP. The uptake was measured at three time points (0.5, 1, and 2 min) for substrate concentrations of 5, 15, and 50 μM, and at two time points (1 and 2 min) for substrate concentrations of 150 and 500 μM. K_m and V_{max} were estimated by the nonlinear least-squares method.

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