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Transnasal Delivery of Methotrexate to Brain Tumors in Rats: A New Strategy for Brain Tumor Chemotherapy

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Abstract: Brain tumors are one of the most lethal and difficult to treat. Their treatment is limited by the inadequate delivery of antitumor drugs to the tumor. In order to overcome this limitation, the possibility of the nose−brain direct transport pathway was evaluated using methotrexate (MTX) as a model antitumor agent. The direct transport of nasal MTX to the cerebrospinal fluid (CSF) was examined by comparing the concentration of MTX in the plasma and the CSF after intraperitoneal (IP) and intranasal (IN) administrations. The brain uptake of MTX was evaluated based on a multiple-time/graphical analysis by measuring the concentration of MTX in the plasma and in the brain. The feasibility of nasal chemotherapy was examined by three nasal dosings of MTX to tumor-bearing rats *in vivo* at two day intervals with peritoneal application as a positive control. MTX showed a significant inhibitory effect on the *in vitro* growth of 9L glioma cells with 50% growth inhibitory concentration at 7.99 ng/mL. The pharmacokinetic studies clarified the significant direct transport of MTX from nasal cavity both to the CSF and to the brain. Nasal chemotherapy with MTX significantly reduced the tumor weight as compared to nontreatment control and IP group. The strategy to utilize the nose−brain direct transport can be applicable to a new therapeutic system not only for brain tumors but also for other central nervous system disorders such as neurodegenerative diseases.

Keywords: Methotrexate; nasal absorption; cerebrospinal fluid; brain delivery; 9L glioma cell; inoculation

Introduction

Brain tumors are one of the most lethal forms of cancer and are extremely difficult to treat.[1] Their treatment is limited by the inadequacy of delivering antitumor agents to the desired targets.[2] In order to treat tumors efficiently, it is necessary to transport antitumor agents across the specialized vascular system of the brain, the blood−brain barrier (BBB). Low permeability of the BBB results in only small lipophilic molecules penetrating well in the absence of a specific transporter; the larger a molecule and the less lipophilic, the lower its permeability across the BBB.[3] Low penetration of chemotherapeutic drugs into the central nervous system (CNS) complicates intracerebral malignancy treatment. Several solutions are currently being explored[4,5] however, no safe, robust, and convenient method to treat brain tumors is currently available that enables brain-penetrating chemotherapy. Intrathecal drug delivery is difficult, uncomfortable and not amenable to a long duration of daily treatment.

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Hyperosmotic BBB disruption is used but is an unpleasant surgical intraarterial procedure with toxic effects and seizure-inducing potential. BBB circumvention by direct intracerebral administration permits increased access to intracerebral tumors by cancer chemotherapeutic drugs with increased survival data that are important and encouraging, but a better chemotherapy delivery system is needed.[6]

Nasal drug administration has been used as an alternative route for the systemic availability of drugs restricted to intravenous administration. This is due to the large surface area, porous endothelial membrane, high total blood flow, the avoidance of first-pass metabolism, and ready accessibility.[7] The nasal administration of drugs, including peptides and protein drugs, for systemic medication has been widely investigated in recent years. Recent studies have shown that the nasal route can also successfully be used to deliver drugs to the CNS.[8,9] The direct anatomical connection between the nasal cavity and the CNS makes it possible to deliver many substances.[10−12] Low molecular weight drugs and peptides directly reach the CNS by circumventing the BBB, which provides the basis for the development of CNS therapeutic agents for intranasal administration. It is the only site in the human body where the nervous system is in direct contact with the surrounding environment.[13,14] Drugs have been shown to reach the CNS from the nasal cavity by direct transport along the olfactory and trigeminal neural pathways.[15] Drugs administered to the CNS by the intranasal route not only circumvent the BBB but also avoid the hepatic first-pass effect. Furthermore, reduction of delivery to nontargeted sites, administration of lower doses and, in turn, reduced toxicity are feasible. Our previous studies have clarified the relation of direct nose−CSF transport with the physicochemical characteristics of drugs, such as molecular weight and lipophilicity,[16−21] and demonstrated that intranasal administration offers a simple, practical, noninvasive and convenient route for rapid drug delivery to the brain/CNS,[22] as well as to the systemic circulation.[23−25]

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In this study, the significance of the direct nose−brain transport pathway for brain tumor chemotherapy is evaluated. Based on the information published in previous reports, MTX, which is a highly hydrophilic and water-soluble drug (Log $P_{n\text{-octanol/water}}$; −1.85)[26] of small molecule size (MW: 454.5 Da), was selected as a model anticancer drug. Additionally, in order to enhance the nose−brain transport of MTX, acetazolamide (AZA), which reduces the clearance of drugs from the CSF by inhibiting CSF secretion from the choroid plexus, and sodium carboxymethyl cellulose (CMC), which is added to the nasal dosing solution to increase the nasal residence time of MTX, were utilized together with nasal MTX. The feasibility of MTX chemotherapy against 9L glioma cells inoculated into the rat brain was evaluated by comparing the effect of nasally administered MTX with that of intraperitoneally administered MTX as a positive control, and a nontreatment group as a negative control.

Experimental Section

1. Materials. MTX was purchased from Sigma-Aldrich (St. Louis, MO). ^{3}H-Methotrexate (^{3}H-MTX, 5-[6-^{3}H]-, specific activity, 555 GBq/mmol) was obtained from PerkinElmer (Waltham, MA). A cell counting kit (WST assay kit) was purchased from DOJINDO Laboratories (Kumamoto, Japan). All other reagents used in this study were reagent grade from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) or Nacalai Tesque, Inc. (Kyoto, Japan).

2. IC_{50} of MTX against 9L Glioma Cells. 9L glioma cells were cultured at 37 °C with DMEM supplemented with 10% fetal bovine serum, 1% L-glutamine, 1% nonessential amino acids and 5% antibiotic−antimycotic mixture in humidified air in a 5% CO_2 atmosphere. Cells were cultured in fresh medium for 48 h before harvest for the assay.

Cells were seeded on a flat-bottomed MICROTEST tissue culture plate with 96 wells and a low evaporation lid (Nippon Becton Dickinson Co., Ltd., Tokyo, Japan) at a density of 1 × 10^3 cells/200 μL/well, and incubated at 37 °C in humidified air in a 5% CO_2 atmosphere for 48 h. The culture medium was replaced with 200 μL of culture medium containing various concentrations of MTX (0, 0.005, 0.111, 0.222, 0.444, 0.888, 1.775, 3.551, 7.102, 14.203, 28.406, 56.813, 113.625 ng/mL) and cultured under the same conditions for 96 h. The viability of cells was determined according to the WST assay (the standard protocol of the cell counting kit).

3. Pharmacokinetic Study. All animal experiments were performed in accordance with the Guideline of Setsunan University for the Care and Use of Laboratory Animals. Male Wistar rats (200−300 g) were anesthetized with intraperitoneal pentobarbital (40 mg/kg, Nembutal; Abbot Laboratories, Abbot Park, IL). The right femoral artery was cannulated for blood sampling.

3.1. Direct Transport of MTX from the Nasal Cavity to the CSF. The trachea and esophagus of rats in the nasal application group underwent surgery as previously reported by Hiral et al.[27] To examine the effect of AZA on direct transport to the CSF, the rat received oral AZA (10 mg/mL, 1 mL/kg) 30 min before the nasal administration of MTX according to the previous publication.[22] MTX was applied to the rat peritoneally (1.25 mg in 0.2 mL of saline) or nasally (2.5 mg in 50 μL of saline, 25 μL bilaterally). The blood was collected at appropriate time intervals. Soon after the final blood sampling, CSF was collected by cisternal puncture, as previously reported by Chou and Levy.[28] Collection was terminated as soon as blood appeared, and only CSF samples that exceeded 150 μL were accepted for analysis. The CSF was divided into 2 parts, an initial and latter half. The concentration of MTX in the latter half was determined.[22]

3.2. Brain Uptake of ^{3}H-MTX after IN and IP Administrations. The trachea and esophagus of rats from the IN application group underwent surgery as described above. The rat then received the dose of ^{3}H-MTX nasally (1850 kBq [3.33 nmol] in 50 μL of saline containing 1 w/v % CMC, 25 μL bilaterally) or peritoneally (925 kBq [1.67 nmol] in 0.2 mL of saline). Blood samples were taken from the right femoral artery. The sampling schedules were as follows: 5, 10, 15, 20, and 30 min for 30 min tissue sampling, and 10, 20, 30, 45, and 60 min for 60 min tissue sampling. The rat was decapitated 30 or 60 min after drug application, and the brain was removed from the skull and washed with saline. Meninges were carefully removed, and the cerebral cortex was dissected.

3.3. Repeated IN and IP Administrations. Rats were divided into six groups, first IN, first IP, second IN, second IP, third IN and third IP. Under intraperitoneal pentobarbital anesthesia, MTX was applied nasally (2.5 mg in 50 μL of saline containing 1 w/v % CMC, i.e. 25 μL bilaterally) to rats in the first IN, second IN and third IN groups with a micropipet. MTX was also applied peritoneally (1.25 mg in 0.2 mL of saline) to rats in the first IP, second IP and third IP groups. The blood was collected from rats in the first IN and first IP groups for 180 min at appropriate time intervals. Two days later, rats in the second IN, second IP, third IN and third IP groups received second IN and IP dosings of MTX as described above. Blood was collected from rats in

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the second IN and second IP groups for 180 min. Two days later, rats in the third IN and third IP groups received the third IN or IP dosing of MTX. Blood was collected from rats in the third IN and third IP groups for 180 min as described above.

3.4. Antitumor Efficacy of Nasal and Intraperitoneal MTX against Inoculated 9L Glioma. 9L rat glioma cells were inoculated into the right frontal cortex of the rat according to the method of Tokunaga et al.[29,30] Briefly, male Fischer rats (200−220 g) were anesthetized with intraperitoneal pentobarbital (40 mg/kg). An incision was made in the skin over the skull to expose the bregma. A 1 mm hole was made 2 mm right and 3 mm rostral from the bregma. The suspension of 9L rat glioma cells (5×10^3 cells/10 μL) was slowly injected at 3 mm depth from the surface of the skull with a microsyringe. After inoculation, the hole was closed with bone wax and the skin was sutured. Four days after inoculation, MTX was administered three times at two-day intervals. At each treatment, AZA (10 mg/mL, 1 mL/kg) was orally administered to the IN group 30 min before MTX administration in order to enhance the brain uptake of MTX.[22] The IP and IN doses were 1.25 mg and 2.5 mg, respectively, at which doses the areas under the curve of the concentration (AUC) in the plasma after IP and IN administrations were similar. In order to increase the viscosity of the solution and hence to enhance the nasal residence time of MTX,[23] 1 w/v % CMC was added to the nasal dosing solution. On day 10, the rat was decapitated, and the brain tumor was isolated and weighed.

4. Analytical Procedure. *4.1. MTX.* MTX was analyzed according to the methods of Assadullahi et al.[31] and So et al.[32] with slight modifications. The plasma was separated by centrifugation of the whole blood at 5,000 rpm for 5 min. Methanol (1.1 mL) was added to 100 μL of plasma and mixed well. The mixture was centrifuged at 10,000 rpm for 10 min, and 1 mL of the supernatant was evaporated to dryness under reduced pressure at 50 °C. The residue was reconstituted in 80 μL of HPLC mobile phase. The sample (30 μL) was injected into a Shimadzu HPLC system (LC-6A; Shimadzu, Kyoto, Japan) coupled with Lichrospher 100RP-18e (5 μm; Merck, NJ) at 50 °C. CSF samples were directly injected into the column without any treatment. The mobile phase was 50 mM phosphate buffer (pH 3.0): acetonitrile (9:1), and the flow rate was 1.0 mL/min. The absorbance was monitored at 313 nm.

4.2. Radioactivity of ^{3}H-MTX. The plasma (100 μL) was transferred to a counting vial and treated with 0.5 mL of Soluene-350 (Packard, Groningen, The Netherlands). The dissected cerebral cortex was transferred to a counting vial and weighed. The tissues were dissolved in 1 mL of Soluene-350. Each sample was neutralized with 5 N HCl, and then 10 mL of Clear-sol I (Nacalai Tesque, Kyoto, Japan) was added. The radioactivity in the plasma and brain tissue was counted with a liquid scintillation counter (LSC3500; Aloka, Tokyo, Japan).

5. Data Calculation. *5.1. Data Analysis.* A computer program, WinNonlin (Pharsight, Cary, NC), was utilized to obtain the concentration of MTX exhibiting 50% *in vitro* growth inhibition of 9L glioma cells (IC_{50}) and AUC of MTX shown in Figure 4. The inhibitory sigmoid E_{max} model and two-compartment model with first-order absorption were employed for IC_{50} and AUC, respectively.

5.2. Analysis of Brain Uptake. The brain uptake of ^{3}H-MTX was quantified based on a multiple-time/graphical analysis developed by Blasberg et al.[33] In brief, the unidirectional influx clearance (CL_{inf}) and the apparent intravascular volume of distribution at time zero (V_i) followed the equation

$$C_{brain(t)}/C_{p(t)} = CL_{inf} \times AUC_t/C_{p(t)} + V_i$$

where $C_{brain(t)}$ and $C_{p(t)}$ are the concentrations of ^{3}H-MTX at time t in the cerebral cortex and in the plasma, respectively. AUC_t is the area under the plasma concentration of ^{3}H-MTX−time curve up to time t. CL_{inf} and V_i were obtained from the slope and the intercept, respectively, of the linear plot of $C_{brain\ (t)}/C_{p(t)}$ against $AUC_t/C_{p(t)}$.

6. Statistical Analysis. Student's *t*-test and Tukey's ANOVA multiple comparison test were used for statistical analysis of the data presented in Figures 2 and 5.

Results

1. *In Vitro* Growth Inhibitory Effect of MTX on Tumor Cells. Figure 1 shows the change of the viability of the 9L glioma as a function of the concentration of MTX in the culture medium. According to the curve, IC_{50} of MTX against 9L glioma was 7.99 ng/mL. This very low value of IC_{50} indicates that MTX exhibited a significant inhibitory effect on the growth of 9L glioma cells. From this finding, MTX is expected to show *in vivo* potent antitumor efficacy.

2. Direct Transport of MTX to the CSF after Nasal Administration. Figure 2 shows the concentration−time profiles of MTX in the plasma and the concentration of MTX

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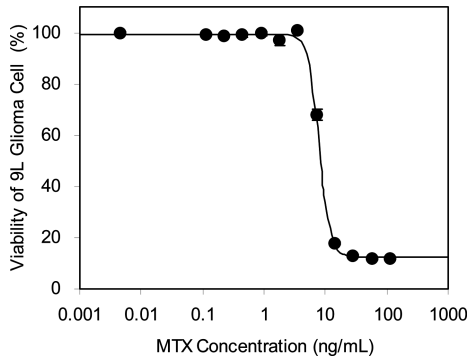

Figure 1. Growth inhibition curve of MTX against 9L glioma cells. Cells were seeded on a 96 well plate at a density of 1×10^3 cells/200 μL/well and incubated for 48 h. MTX were added to the culture medium at appropriate concentrations (0−114 ng/mL), and 9L glioma cells were cultured for 96 h. The viability of cells was determined according to the WST assay.

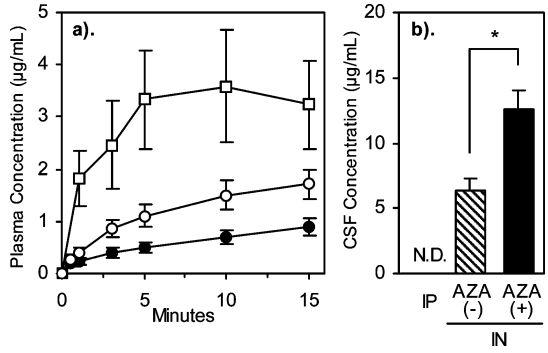

Figure 2. The plasma concentration−time profiles (a) and the concentrations in the CSF (b) after intraperitoneal and nasal administrations of MTX. MTX was applied to the rat peritoneally (1.25 mg in 0.2 mL of saline, $n = 4$; □ and N.D.). MTX was also applied nasally without pretreatment of AZA (2.5 mg in 50 μL of saline, 25 μL bilaterally, $n = 3$; ○ and hatched bar). For the examination of the effect of AZA on the direct transport to the CSF, the rat received oral AZA (10 mg/mL, 1 mL/kg) 30 min before the nasal administration of MTX (2.5 mg in 50 μL of saline, 25 μL bilaterally, $n = 3$; ● and solid bar). The CSF was taken 15 min after dosing of MTX. Data represent mean ± SE. N.D.: not detected. Student's *t*-test was applied on the analysis of the CSF concentration of MTX after the nasal administration with and without pretreatment of AZA (*$p < 0.05$).

in the CSF at the end of the study following IP and IN administrations. The concentration in the plasma following IP dosing quickly increased and decreased with time and MTX was not detected in the CSF at the end of the study. In contrast, the concentrations in the plasma following IN application gradually increased. The concentration in the CSF 15 min after administration was 6.42 ± 0.91 μg/mL, which is 3-fold higher than in the plasma just before CSF sampling. These findings clearly indicate the significant direct transport of MTX from the nasal cavity to the CSF. The effect of AZA on the direct

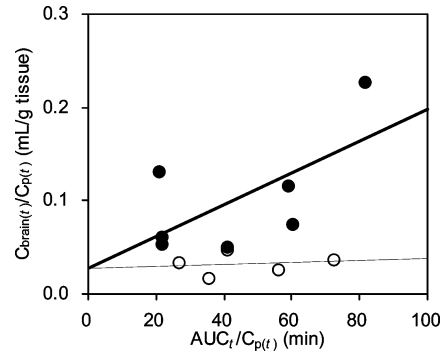

Figure 3. The integration plot of MTX following intraperitoneal and intranasal applications. ^{3}H-MTX was dissolved in saline, and administered intraperitoneally (925 kBq [1.67 nmol] in 0.2 mL of saline; $n = 5$, ○). ^{3}H-MTX was also dissolved in saline containing 1 w/v % CMC, and administered nasally (1850 kBq [3.33 nmol] in 50 μL of saline, 25 μL bilaterally; $n = 6$; ●). After collecting blood sample, the rat was decapitated 30 or 60 min after drug application, and the cerebral cortex was dissected. Each point represents the data from an individual animal.

transport of MTX to the CSF is indicated in Figure 2. Oral AZA slightly decreased the concentration in the plasma. It is noteworthy that the concentration of MTX in the CSF was markedly increased to 12.54 ± 1.54 μg/mL, which was 195% in comparison with that without AZA treatment.

3. Comparison of the Brain Uptake of ^{3}H-MTX between Nasal and Peritoneal Application. To confirm whether MTX was directly transported from the nasal cavity not only to the CSF but also to the brain parenchyma, the brain uptake of MTX was analyzed. The concentrations in the plasma and the cerebral cortex were determined following IP and IN administrations of ^{3}H-MTX in the absence of AZA and evaluated based on a multiple-time/graphical analysis. A graphical presentation of the result is shown in Figure 3. According to the theory developed by Blasberg et al.,[33] the slope and the intercept of the line in the plot represent CL_{inf} and V_i, respectively. Linear regression analysis of the data clarified that CL_{inf} following IP and IN administrations was 0.11 μL/min/g tissue and 1.71 μL/min/g tissue, respectively. CL_{inf} after IN dosing showed a one-order magnitude higher value. V_i was similar for both groups (26.8 μL/g tissue after IP dosing and 26.7 μL/g tissue after IN dosing).

4. Effect of Repeated Applications on the Nasal Absorption and Disposition of MTX. To investigate the effect of multiple applications of MTX on the absorption and disposition of MTX, repeated IN and IP administrations were performed (Figure 4). Following IP application, the profile showed an initial rapid increase with maximum concentration at 20 min and decreased thereafter. In contrast, the initial increase in the plasma concentration was slow and the peak concentration was low following IN application. No significant difference was observed in the AUC after IP and IN administration. The profiles and AUCs after second application were similar to those after the first application; however, the concentrations after the third IN and IP applications were

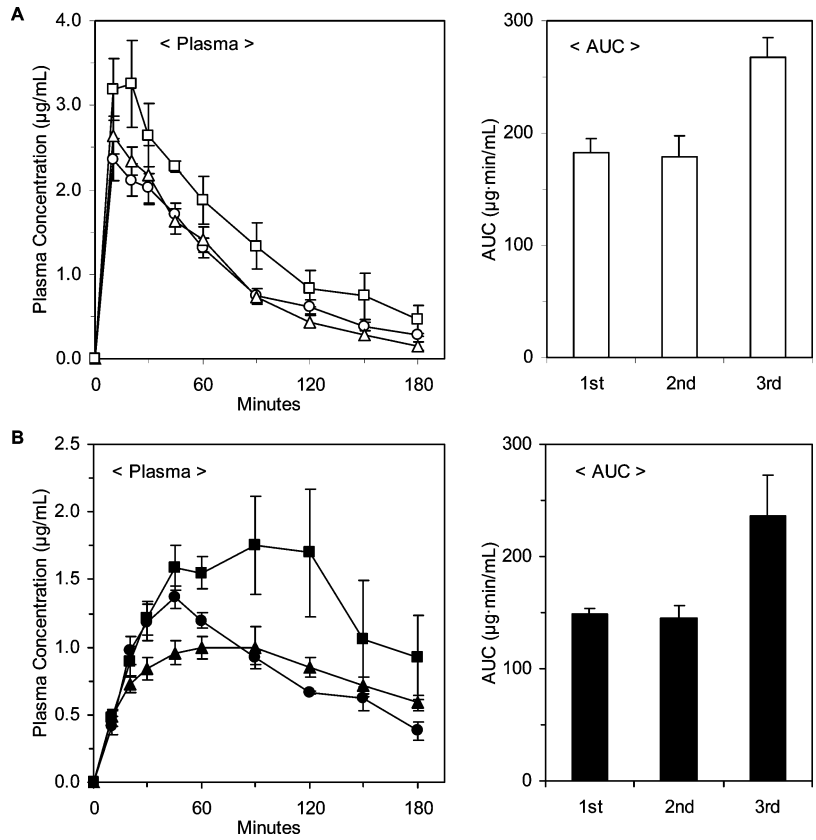

Figure 4. The plasma concentration−time profiles and AUC of MTX following repeated intraperitoneal (A) and nasal (B) application. MTX was dissolved in saline and administered intraperitoneally (1.25 mg in 0.2 mL of saline; $n = 4$; first application, ○; second application, Δ; third application, □). MTX was also dissolved in saline containing 1 w/v % CMC and administered nasally (2.5 mg in 50 μL of saline, 25 μL bilaterally; $n = 4$; first application, ●; second application, ▲; third application, ■). Data represent mean ± SE.

higher than after the first and second applications. AUCs after the third IP and IN applications showed 50% and 60% higher values, respectively, than the second application. The change in the profiles and AUCs after the third application suggests that this change may be due not to the increase in absorption but to the decrease in the elimination of MTX.

5. Antitumor Efficacy of MTX against the Brain Tumor. Tumor-bearing rats were treated with nasal or peritoneal MTX three times at two-day intervals. The tumor weights 2 days after the third treatment are indicated in Figure 5. Inoculated 9L glioma cells grew to a 300 mg tumor over the 10 days from inoculation. Peritoneal MTX inhibited tumor growth by 20%. In contrast, significant inhibition of tumor growth was observed after nasal treatment with MTX. Tumor weight was 60 mg, which was 20% and 25% of those of the control and IP groups, respectively.

Discussion

For the past two decades, many reports regarding nasal drug delivery to the CSF and the brain have been published. Not only metal ions[34−36] and small molecules[16−22] but also peptides,[37] proteins[14,15] and gene treatment[38,39] as well as viruses[40,41] and cells[42] can be delivered from the nasal cavity to the brain. Such approaches provide an effective means of relieving migraines,[43,44] improving memory[45,46] and treating other

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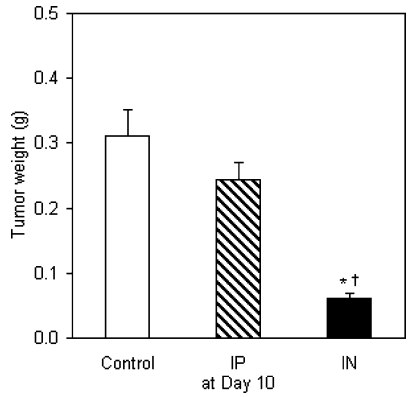

Figure 5. The tumor weight of rats receiving 3 doses of MTX 10 days following inoculation. 9L rat glioma cells were inoculated into the right frontal cortex of male Fischer rats. Four days after inoculation, MTX was administered peritoneally (IP; $n = 7$; hatched bar) or nasally (IN; $n = 8$; solid bar) three times at two day intervals. At each treatment, AZA (10 mg/mL, 1 mL/kg) was orally administered to the IN group 30 min before MTX administration. The IP and IN doses of MTX were 1.25 mg and 2.5 mg. CMC was added to the nasal dosing solution at the concentration of 1 w/v %. On day 10, the rat was decapitated, and the brain tumor was isolated and weighed. Data represent mean ± SE. The statistical analysis was done on the data using Tukey ANOVA multiple comparison test. According to the test result, statistically significant differences were observed between the control and IN groups ($^*p < 0.001$), and between the IP and IN groups ($^\dagger p < 0.001$).

cerebrosis.[47−49] These advantages have highlighted intranasal delivery in the exploration of brain drug targeting systems.

According to some studies, highly permeable drugs are likely absorbed preferentially by the systemic circulation and no significant transport from the nasal cavity to the CSF and the brain was observed. For example, Hussain et al.[50] reported that no significant difference was observed in the AUC ratio (AUC_{brain}/AUC_{plasma}) after nasal and intravenous administrations of a cognition enhancer. According to Van den Berg et al.,[51] AUC_{CSF}/AUC_{plasma} ratios of hydroxocobalamin after nasal and intravenous applications showed the same values in humans and rats. In our preliminary study, a lipophilic anticancer drug, mitomycin C, showed no significant transport from the nose to the CSF (data not shown). Based on these reports and our previous findings, hydrophilic MTX was selected as a model antitumor drug. As expected, MTX showed a higher concentration in the CSF after nasal administration while MTX was not detected in the CSF after IP administration. Solubility in water is also important since the dosing volume to the rat nasal cavity is limited (usually, less than 50 μL). The solubility of MTX was sufficiently high for animal experiments in this study.

A significant antitumor effect of nasal MTX was confirmed in an *in vivo* animal study compared to peritoneal MTX. As shown in Figure 1, IC_{50} against the *in vitro* growth of 9L glioma cells was 7.99 ng/mL. It is indicated in Figure 2 that CSF levels after nasal application were significantly higher than after intraperitoneal application, despite much lower plasma levels after nasal application. These findings agree with the report by Wang et al.[52] Additionally, the concentration in the CSF after nasal administration was 12.54 ± 1.54 μg/mL under the influence of oral AZA, which was 3-fold higher than IC_{50}. These results suggest that MTX is directly transported from the nasal cavity to the CSF and that the concentration in the CSF is high enough to inhibit the *in vivo* growth of 9L glioma. However, high exposure to MTX by the normal brain might cause an unexpected side effect. Short-term exposure and coadministration of leucovorin and folic acid might help to reduce MTX toxicity in clinical therapy. In the examination of *in vivo* antitumor efficacy, 9L glioma cells were inoculated in the frontal cerebral cortex. As mentioned in the previous paper,[21] the distribution of directly transported drugs is not uniform in the CSF; the closer to the nasal cavity that the CSF is, the higher

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the drug concentration in the CSF; therefore, the olfactory bulb is suitable for tumor cell inoculation, although the olfactory bulb is small and the space for tumor growth is limited. The frontal cortex is the next closest to the olfactory bulb, which is why the frontal cortex was selected for tumor cell inoculation.

For antitumor efficacy of nasal MTX, a higher concentration of MTX in the CSF is important. In order to enhance the efficacy of nasal MTX, AZA was utilized in this study. One of the important factors to determine the concentration of the drug in the CSF is its clearance by the bulk flow of the CSF. Drug clearance can be decreased by inhibition of the CSF secretion rate. Slow bulk flow results in a higher CSF concentration of drugs directly transferred from the nasal cavity.[22] AZA is a diuretic with an inhibitory effect on carbonic anhydrase. Through the inhibition of carbonic anhydrase in the choroid plexus, AZA decreases the secretion of CSF. de Lange et al. reported that CSF drug levels do not always correlate well with parenchymal brain levels and in some cases are very different;[53] however, as we previously reported, AZA increased the direct transport of 5-fluorouracil to the CSF and the brain from the nasal cavity.[22] The same effect is expected in nasal MTX treatment for brain tumors. At the same time, AZA is expected to reduce the systemic side effects of MTX. As shown in Figure 2, the concentration of MTX in the plasma after nasal administration was decreased by oral AZA pretreatment. AZA is known to increase the urinary pH by inhibiting carbonic anhydrase in the kidney. Since MTX is an acidic drug, renal tubular reabsorption from the basic urine is decreased. The lower concentration in the plasma under the influence of AZA is the result of enhanced renal excretion.

A drug applied to the nasal cavity is translocated, together with the dosing solution, to the nasopharynx and then to the gastrointestinal tract by the coordinated beating of the cilia of some nasal epithelial cells.[23−25] A long nasal residence time leads to a long duration of the MTX concentration in the CSF. In order to enhance the duration of the concentration in the CSF, the translocation of the nasally applied drug to the gastrointestinal tract should be controlled. For this purpose, CMC was added to the nasal dosing solution at a concentration of 1 w/v %, and likely contributed to enhance the antitumor efficacy of MTX after nasal application.

Since MTX is toxic to nontumor normal cells, multiple applications are likely to change the nasal absorption and/or disposition of MTX. In order to examine this possibility, the effect of multiple dosings on the concentration−time profiles of MTX in naive rats following repeated applications was determined (Figure 4). The application schedule was the same as that of the study on *in vivo* antitumor efficacy. In first and second applications, no significant changes in the profiles and AUC following IN and IP applications were observed; however, the concentration of MTX was high and consequently AUC was significantly increased after the third IN and IP applications. The fact that the change and its degree were common to IN and IP applications suggest that this may not be due to increased absorption but to decreased elimination. The main elimination pathway of MTX is renal excretion. Side effects of MTX are often observed in the kidney. Since the solubility of MTX is markedly dependent on the pH of the vehicle, MTX might be recrystallized in renal tubules at the acidic urinary pH. Two previous MTX administrations could cause renal dysfunction, resulting in an increased concentration of MTX and AUC after the third IN and IP applications; therefore, the change in the elimination process of MTX had the same effect on both IN and IP groups in the study on *in vivo* antitumor efficacy.

MTX, which indicates a marked growth-inhibitory effect on 9L glioma cells, is transported directly from the nasal cavity to the CSF and the brain. MTX is a folic acid antagonist that has been extensively used as an anticancer agent, but its effectiveness against brain tumors has been limited. MTX is significantly protein bound in plasma,[54] and there is evidence from microdialysis studies that only the free fraction of the drug is capable of significant entry into bulk tumor, an area where the BBB is typically compromised. The penetration of MTX into areas where the BBB is intact, such as the normal brain and outer regions of tumors, such as gliomas, is notably poor even for the free drug.[55,56] Intranasal administration potentially allows direct transport from the nose to the brain. Nasal treatment of tumor-bearing rats by MTX was successful, reducing the tumor weight significantly. The strategy of utilizing nose−brain direct transport is expected to be applicable to a new therapeutic system not only for brain tumors but also for other central nervous system disorders, such as neurodegenerative diseases.

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