NOTE *Pharmacology*

Effect of cyclosporin on distribution of methotrexate into the brain of rats

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ABSTRACT. The effect of the antitumor drug, methotrexate (MTX), which is applied to brain tumors, is restricted by the blood-brain barrier (BBB), which is composed of P-glycoprotein (P-gp) and multidrug resistance associated protein (MRP). We, therefore, studied if a potent P-gp and MRP modulator, cyclosporin A (CysA), can modulate the MTX concentration in the rat brain. If it can, which route is more effective, intravenous or intrathecal? We intravenously or intrathecally administered MTX to rats with or without CysA. After 6 hr, brains and cerebrospinal fluid (CSF) were sampled, and their MTX concentrations were compared. Each MTX concentration was determined by high-performance liquid chromatography with UV detection. CysA had no significant affect on the MTX concentration in the brain or CSF when MTX was intravenously injected. In contrast, when MTX was intrathecally administered, CysA had a larger effect on the MTX concentration in the brain than in the CSF. This indicates CysA potentiated the brain MTX concentration when MTX was intrathecally administered. It is suggested that CysA did not potentiate the distribution of MTX from blood into the brain, but instead potentiated the distribution of MTX from CSF into the brain. Since chemicals in CSF generally diffuse into the brain easily, CysA probably inhibited the excretion of MTX from the brain. This could be caused by inhibition of P-gp or MRP at the BBB. Therefore, CysA can be a useful tool to achieve an appropriate MTX concentration in brain.

KEY WORDS: cyclosporine A, intrathecal administration, methotrexate, P-glycoprotein

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Methotrexate (MTX), which is prescribed for brain tumors, is strongly prevented from entering the brain by the blood-brain barrier (BBB) and blood-CSF barrier (choroid plexus). Though various methods have been developed to achieve an effective MTX concentration, their effects have been limited [1, 4, 7, 9, 13].

P-glycoprotein (P-gp) and multidrug resistance associated protein (MRP) are major organic transporters responsible for preventing xenobiotics from entering the brain through the BBB [5, 11].

The common characteristics of the P-gp substrates are identified so far are as follows: (1) planar structure, (2) high lipophilicity and (3) neutral of positive charge. So, MTX is unlikely to be a substrate for P-gp, because MTX is water soluble and mainly transported by the reduced folate carrier (RFC). However, some studies suggest that MTX can be a substrate for P-gp and MRP under certain circumstances [3, 6].

Therefore, we examined if a potent P-gp and MRP modulator, cyclosporine A (CysA), can potentiate the MTX concentration in the rat brain.

Male Sprague-Dawley rats (9 weeks old, weighing between 286 and 316 g) were used in this study. MTX (2 mg/body) and CysA (5 mg/body) were injected into the animals by an intravenous (i.v.) or intrathecal (i.t.) route. Based on the route of drug administration, the animals were divided into 5 groups as follows: group Miv, MTX (i.v.)+saline (i.t.); group Mit, MTX (i.t.); group Miv+Civ, MTX (i.v.)+CysA (i.v.)+saline (i.t.); group Mit+Civ, MTX (i.t.)+CysA (i.v.); and group Mit+Cit, MTX (i.t.)+CysA (i.t.). All administrations were performed under anesthesia with pentobarbital (50 mg/kg, intraperitoneally). To avoid increasing intracranial pressure, i.t. injections were performed after removing as much CSF as possible.

The rats in each group were euthanized at 6 hr after administration in order to collect CSF and brains (n=5, respectively). The CSF (0.1 m*l*) was sampled from the cisterna just before euthanasia. The isolated brains were stored at −80°C until used for analysis of MTX after being rinsed out.

Each brain was homogenized with methanol (20 m*l*) to extract MTX and MTX polyglutamates. Then, the homogenate was centrifuged at 3,000 g for 20 min to separate the clear liquid layer and the residue. The clear layer was evaporated, and the residue was mixed with 10 mM sodium acetate buffer (pH 1.6, 50 m*l*) to dissolve the water-soluble polyglutamates of MTX. Then, the mixture was centrifuged at 3,000 g for 20 min. The obtained clear liquid layer was mixed with the one obtained before, and the mixture was evaporated until dry.

In order to clean up and concentrate MTX and its polyglutamates, solid phase extraction (Sep-Pak® C18 Plus cartridge, Waters Corporation, Milford, MA, U.S.A.) was applied to the mixture. Then, MTX and MTX polyglutamates

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were eluted with 2 m*l* of 50% methanol solution (pH 7.0), and the elute was subjected to HPLC analysis of MTX.

$HClO_4$ (0.2 m*l*) was added to CSF samples (0.1 m*l*) for the removal of protein, and the samples were then centrifuged at 12,000 g for 2 min. The obtained supernatant was subjected to HPLC analysis of MTX.

MTX and its polyglutamates were analyzed by an HPLC system. The mobile phase consisted of 10 mM acetate buffer (pH 1.6) and acetonitrile (90:10, v/v), and the effluent was monitored by a UV detector (SPD-6A, Shimadzu, Kyoto, Japan) at wavelength of 313 nm. The analytical column was ion-exchange column (Partisil® 10 SCX, 4.6 mm × 250 mm, Whatman, part of GE Healthcare, Tokyo, Japan). The CSF and the brain samples were examined for MTX and its polyglutamates, and combined concentrations were expressed as total MTX. However, MTX pentaglutamate (MTX-glu5) and hexaglutamate (MTX-glu6) were not analyzed in the brains, because there were negligible amounts in a preliminary study. The recoveries of monoglutamate, diglutamate triglutamate and tetraglutamate of MTX were 93.2 ± 4.1%, 79.2 ± 5.5%, 72.7 ± 4.0% and 70.2 ± 1.7%, respectively (n=5) at 1 μg/m*l*. The coefficients of variation (CV) for them were 4.4, 6.9, 5.5 and 2.5, respectively. The interday CV values in the assay ranged from 1.6 to 5.8% with a limit of quantification of 7.7 nM at a signal-to-noise ratio of 3 (n=5).

Data are displayed as means±SD. Differences in mean values between the groups were analyzed by Scheffe's multiple comparison test after one-way ANOVA. Equal variances among the groups were confirmed by Bartlett test. Differences were considered significant at $P<0.05$.

Each group's MTX concentrations in the brain and CSF are shown in Fig. 1. Between the intravenous administration groups, Miv and Miv+Civ, there were no significant differences in MTX level in both the brain and CSF.

The intrathecal administration groups, Mit, Mit+Civ and Mit+Civ, naturally tended to show higher MTX levels in the CSF than the intravenous administration groups, Miv and Miv+Civ. However, the Mit+Civ and Mit+Cit groups revealed significantly higher MTX levels in the brain than the Mit group.

Table 1 shows the ratio of MTX levels between the brain and CSF. While the Mit group showed much lower MTX levels in the brain than in the CSF, the Mit+Civ and Mit+Cit groups showed much higher MTX levels in the brain than in the CSF.

In summary, we found the following: CysA barely helped MTX penetrate into the brain and CSF. However, when MTX was intrathecally administered and CysA was either intravenously or intrathecally administered, CysA potentiated the MTX level in the brain. For the possible mechanism by which CysA potentiated the brain MTX level, the following possibilities may be considered: 1) it may be the result of an increased MTX level in the CSF or 2) CysA may have inhibited MTX exclusion from the brain.

In general, chemicals in CSF easily diffuse into the brain. However, Brain/CSF ratio is quite different among the groups. The quite low ratio of the Mit group suggests that distribution of MTX from the CSF into the brain is relatively

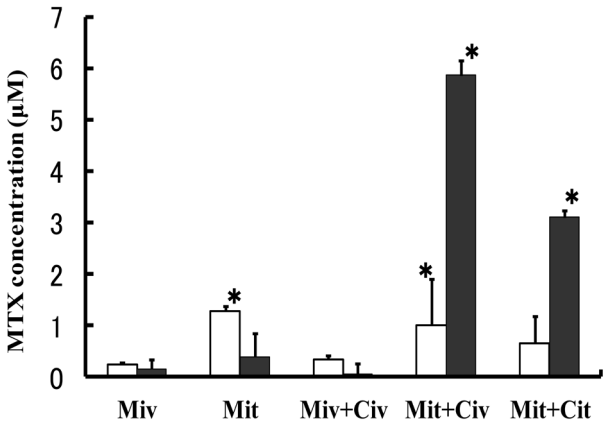

Fig. 1. MTX concentrations in the brain (black column) and CSF (white column) at 6 hr post administration of MTX with or without CysA in rats. Miv group, MTX (i.v.)+saline (i.t.); Mit, MTX (i.t.); Miv+Civ group, MTX (i.v.)+CysA (i.v.)+saline (i.t.); Mit+Civ group, MTX (i.t.)+CysA (i.v.); and Mit+Cit group, MTX (i.t.)+CysA (i.t.). MTX and CysA are abbreviations for methotrexate and cyclosporin A, respectively. Each column and vertical bar show the mean and SD, respectively (n=5). The doses of MTX and CysA were 2 and 0.2 mg/body, respectively. *The concentrations of the Mit+Civ and Mit+Cit groups were significantly higher than those of the other 3 groups ($P<0.05$, Scheffe's multiple comparison test).

Table 1. MTX concentration ratio between the brain and CSF at 6 hr post administration of MTX with or without CysA in rats

Group	Brain/CSF
Miv	0.87 ± 0.42
Mit	0.31 ± 0.14
Miv + Civ	0.25 ± 0.25
Mit + Civ	6.17 ± 2.06
Mit + Cit	5.04 ± 2.03

Miv group, MTX (i.v.)+saline (i.t.); Mit, MTX (i.t.); Miv+Civ group, MTX (i.v.)+CysA (i.v.)+saline (i.t.); Mit+Civ group, MTX (i.t.)+CysA (i.v.); and Mit+Cit group, MTX (i.t.)+CysA (i.t.). MTX and CysA are abbreviations for methotrexate and cyclosporin A, respectively. Each value is presented as the mean±SD (n=5). The doses of MTX and CysA were 2 and 0.2 mg/body, respectively. *The ratios of the Mit+Civ and Mit+Cit groups were significantly higher than those of the other 3 groups ($P<0.05$, Scheffe's multiple comparison test).

slow and therefore suggests that high MTX levels were not caused mainly by diffusion from the CSF in the Mit+Civ and Mit+Cit groups. The similar MTX concentrations in the CFS among the Mit, Mit+Civ and Mit+Cit groups may suggest that CysA did not significantly inhibit the blood-CSF barrier. Therefore, it is considered that CysA inhibited MTX exclusion from the brain and resulted in quite high MTX concentrations in the brain. This indicates that CysA might work as a P-gp or MRP modulator at the BBB.

In our preliminary study, when MTX was intravenously or intrathecally administered to rats, the half-lives of the plasma concentrations were approximately 40 and 90 min,

respectively. In order to exclude the possibility of contaminating brains with MTX in blood as long as possible, samples were taken at 6 hr after the administration of MTX in this study. The elimination phase of plasma MTX appeared much earlier than this sampling time, suggesting that our present results were obtained after the distribution equilibrium of MTX was attained between plasma and the brain.

In conclusion, this study indicated that CysA can be an available tool to maintain intrathecally administered MTX at an effective concentration in the brain for a longer time without increasing systemic toxicity, because intrathecal MTX administration requires a lower dose compared with intravenous administration to achieve an appropriate concentration in the brain. Additionally, there may be another advantage to the intrathecal MTX therapy with CysA. It is known that P-gp and MRP also play an important role in intrinsic and acquired multidrug resistance (MDR) in a lot of cancers [2, 8, 10, 12]. The purpose of cancer chemotherapy with a P-gp or MRP modulator for MDR tumors, such as feline CNS lymphoma, is to maintain anticancer drugs at adequate concentrations in tumor cells for a longer time. If CysA effectively works as a P-gp or MRP modulator, we can expect CysA to modulate MDR.

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