**Regular Article**

**In vivo-In vitro Relationship of Methotrexate 7-Hydroxylation by Aldehyde Oxidase in Four Different Strain Rats**

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**Summary:** The in vivo metabolism of methotrexate (MTX) to 7-hydroxymethotrexate (7-OH-MTX) was studied using four strains of rats. When MTX was administered to these rats, 7-OH-MTX was detected as the major in vivo metabolite, mainly in bile and feces, and also slightly in the urine. There were marked strain differences in the amounts of 7-OH-MTX excreted in bile, feces and urine. The highest recovery of 7-OH-MTX in bile, feces and urine was observed in Sea:SD rats (6.2%, 4.2% and 0.8% of dose, respectively), followed by Jcl:SD and Crj:SD rats. The lowest recovery (0.02%, 0.2% and 0.003%, respectively) was observed in WKA/Sea rats. The variations of excreted amount of 7-OH-MTX were closely correlated with the strain differences of cytosolic MTX 7-hydroxylase and benzaldehyde oxidase activities. Our results indicate that variation of formation of 7-OH-MTX from MTX in vivo in rats is due primarily to variation of aldehyde oxidase.

**Key words:** methotrexate; 7-hydroxymethotrexate; aldehyde oxidase; in vivo metabolism; rat strain difference

**Introduction**

Methotrexate (MTX, 4-amino-N10-methylpteroyl-L-glutamic acid) is a potent inhibitor of dihydrofolate reductase, causing depletion of the intracellular tetrahydrofolic acid pool. MTX is widely used in the treatment of acute lymphocytic leukemia in children, as well as for other adult and childhood malignancies, including squamous carcinoma of the head and neck, lymphomas, osteosarcomas, and choriocarcinomas. It has also become a standard therapy for rheumatoid arthritis. However, side effects and drug resistance are serious problems. MTX resistance may contribute to treatment failure. MTX accumulation during therapy is also a serious problem. MTX is metabolized to 7-hydroxymethotrexate (7-OH-MTX), as well as polyglutamates of MTX and 7-OH-MTX, and these polyglutamates are further hydrolyzed by γ-glutamyl hydrolase in humans and various animal species. MTX polyglutamate is reported to inhibit dihydrofolate reductase and other enzymes involved in purine synthesis. MTX is also degraded to 2,4-diamino-N10-methylpteroic acid by intestinal bacteria (Fig. 1). 7-OH-MTX, a major metabolite of MTX, was considered to be a detoxication product. However, it was recently reported that the metabolite induces acute renal and hepatic toxicity, and it has been demonstrated to influence cellular entry, polyglutamation, and efflux of the parent compound in vitro. This metabolite may interact with MTX at a membrane transporter.

It has been reported that aldehyde oxidase functions as an MTX-hydroxylase. Most in vitro studies of the conversion of MTX to 7-OH-MTX have been carried out in rabbits. In this species, several tissues have significant capacity for 7-OH-MTX formation and the liver seems to have a particularly high activity due to the action of aldehyde oxidase (EC 1.2.3.1). Recently, we provided evidence that MTX was mainly oxidized to 7-OH-MTX by aldehyde oxidase, based on strain differences of the enzyme activity in rat liver. We had previously reported a significant strain difference of aldehyde oxidase activity in rat liver. Variations of methotrexate 7-hydroxylase activity in liver cytosol among several strains of rats were closely correlated
Fig. 1. Metabolic pathway of methotrexate.

with those of aldehyde oxidase activity. However, a contribution of xanthine oxidase to the 7-hydroxylation of MTX, in addition to aldehyde oxidase, was observed in human liver. In contrast, Kuroda et al. reported that aldehyde oxidase catalyzes 7-hydroxylation of MTX in monkeys in vivo, but not in rats. Yu et al. suggested that aldehyde oxidase was not the predominant hydroxylating enzyme for MTX in the rat, based on the fact that the excretion ratio of 7-OH-MTX in the bile of rats dosed with MTX was not influenced by coadministration of an inhibitor of aldehyde oxidase. The enzyme responsible for 7-hydroxylation of MTX in vivo in rats remains to be definitively identified.

Strain differences in hydroxylating activity toward MTX in rats could be a useful tool to examine the role of aldehyde oxidase in the metabolism of MTX in this species in vivo. In this study, we investigated the variability of the amounts of excreted 7-OH-MTX using four strains of rats in order to clarify the role of the enzyme as an MTX hydroxylase in vivo in rats is due primarily to variation of aldehyde oxidase.

Materials and Methods

Chemicals: Materials were obtained from the following sources: 1-methylxanthine and 1-methyluric acid from Sigma Chemical Co. (St. Louis, MO, USA); benzoic acid and benzoic acid from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). MTX and 7-OH-MTX were kindly donated by Medical Research Laboratories, Lederle Japan (Tokyo, Japan).

Animals: Four different strains of male rats (6–7 weeks age) were used. Jcl:SD are Sprague-Dawley strains. The animals were housed in cages at 22°C with a 12-h light/dark cycle, with free access to tap water and a standard pellet diet, MM-3 (Funabashi Farm, Funabashi, Japan). The bile ducts of some rats were cannulated with polyethylene catheters. The cannulated rats were immediately transferred to restraining cages for experiments.

Liver preparations: Livers were excised and homogenized in four volumes of 1.15% KCl. The cytosolic fraction of rat livers was obtained from the homogenate by successive centrifugation at 9,000 x g for 20 min and 105,000 x g for 60 min.

Assay for MTX-hydroxylating activity: The incubation mixture consisted of 0.2 μmol of MTX and cytosol equivalent to 50–100 mg of liver in a final volume of 1 mL of 0.1 M K,Na-phosphate buffer (pH 7.4). The incubation was performed at 37°C for 2 hr. After incubation, 20 μg of benzamide (an internal standard) and 2 volumes of acetonitrile were added and the whole was centrifuged. The supernatant was evaporated to about 0.5 mL, and then 10 μL of the solution was subjected to analysis by high-performance liquid chromatography (HPLC) in a Hitachi L-6000 chromatograph (Tokyo, Japan) fitted with an Inertsil ODS-3 column (150 mm × 4.6 mm, GL Sciences, Tokyo, Japan). The mobile phase was acetonitrile-0.1 M KH₂PO₄ (15:85). The chromatograph was operated at a flow rate of 0.3 mL/min at ambient temperature and with a detection wavelength of 254 nm. The elution times of MTX, 7-OH-MTX and benzamide were 15.3, 22.2 and 30.7 min, respectively.

Assay for benzaldehyde oxidase activity: Benzaldehyde oxidase activity was assayed by the method of Johns. The decrease in absorption at 249 nm consequent upon oxidation of benzaldehyde to benzoate was monitored in 165 mM K,Na-phosphate buffer (pH 7.8) at 25°C, using an extinction coefficient of 17.54 mM⁻¹ cm⁻¹.
**In vivo Metabolism of Methotrexate in Rats**

Fig. 2. Methotrexate (MTX) hydroxylase activity in liver cytosol from several rat strains and its correlation with aldehyde oxidase levels. (A) MTX hydroxylase activity. (B) Western blots probed with anti-rat aldehyde oxidase antibody for rat liver cytosol. (C) Correlation between MTX hydroxylase activity in rat liver cytosol and the density of bands for aldehyde oxidase in Western blots.

The each bar represents the mean ± SD of four rats. MTX hydroxylase activity in liver cytosol was assayed by measuring the amount of 7-OH-MTX formed using HPLC. Blots were loaded with 5 μg of protein of rat liver cytosol. Other details are described in Materials and Methods.

**Western blot analysis of rat liver aldehyde oxidase:**

The levels of aldehyde oxidase protein were determined by Western blot analysis of liver cytosolic protein from four strains of rats. Rat liver cytosolic proteins (5 μg) were separated by SDS-polyacrylamide gel electrophoresis (7.5% gel) and transferred to polyvinylidene fluoride membranes (0.2 mm; Bio-Rad, Hercules, CA, USA) by electroblotting. Membranes were then incubated with 5% skimmed milk in 25 mM tris-buffered saline (pH 7.6)-0.1% Tween 20 for 1 hr and probed with a rabbit anti-rat aldehyde oxidase (1:1000) for 3 hr. After washing, antibody binding was detected with horseradish peroxidase-conjugated goat anti-rat IgG, followed by development with ECL Plus (Amersham Pharmacia Biotech, Buckinghamshire, England). The density of the bands was scanned and visualized using Mac BAS Ver. 2.5 software.

**Detection of 7-OH-MTX and MTX in urine, feces and bile of rats:** MTX was given intraperitoneally to rats at a single dose of 50 mg/kg body weight dissolved in saline-1 N NaOH (500:1). The control rats were given the vehicle only. The amounts of MTX and 7-OH-MTX in urine (1 mL) or bile (0.5 mL) were determined by HPLC. The feces (1 g) were dried, pulverized in a mortar and extracted twice with 10 volumes of methanol by sonication and shaking for 20 min each. The combined extracts were passed through a Florisil (Wako Pure Chemical Industries Ltd., Osaka, Japan, 5 × 30 mm) column. The amounts of MTX and 7-OH-MTX excreted in urine, feces and bile were determined by means of solid-phase extraction and HPLC with post-column photo-degradation and fluorometric detection, as reported by Beck et al.25 Briefly, samples were diluted with 50 mM phosphoric acid, and applied to a solid-phase extraction cartridge (Bond Elut Certify II, Varian Sample Preparation Products Inc., Lake Forest, CA, USA). The amounts were determined by HPLC with fluorometric detection (excitation 350 nm and emission 435 nm). HPLC was performed in a chromatograph fitted with a 150 × 4.6 mm Inertsil ODS-3 column and a post-column photochemical reactor (PHRED, Supelco, Bellefonte, PA, USA) equipped with a 254 nm lamp plus a reaction coil (5 m × 0.25 mm i.d.). The mobile phase was 0.01 M Na,K-phosphate buffer (pH 6.5)-N,N-dimethylformamide-30% H_2O_2 (940:60:2). The chromatograph was operated at a flow rate of 1.0 mL/min. The elution times of MTX and 7-OH-MTX were 10.8 and 19.9 min, respectively.

**Results**

**Strain differences of MTX-hydroxylase activity in rat liver:** When the activity for conversion of MTX to 7-OH-MTX was assayed in liver cytosols from four strains of rats, marked variability of the activity was found. Among the strains tested, the highest activity was observed with Sea:SD rats, followed by Jcl:SD and Crj:SD. The lowest activity was seen with WKA Sea rats. The difference in the activity between Sea:SD and WKA Sea strains was about 46-fold (Fig. 2A). Furthermore, Western blot analysis was performed using aldehyde oxidase antibody to estimate the aldehyde oxidase level in liver cytosol. The observed density of the band correlated well with MTX hydroxylase activity except the densities observed in those of Crj:SD and WKA/Sea rats, indicating that MTX hydroxylase activity was exhibited by aldehyde oxidase (Fig. 2B and 2C). The variability closely resembled that of the MTX hydroxylase activity. These facts suggest that the strain
difference of MTX 7-hydroxylase activity in rat livers is due to the quantitative difference of aldehyde oxidase present in the liver cytosolic fraction.

**Excretion of 7-OH-MTX into the bile of Sea:SD and WKA/Sea rats after MTX administration:** The in vivo metabolism of MTX in rats was compared in Sea:SD and WKA/Sea strains of rats. When MTX was administered intraperitoneally to bile-drained Sea:SD rats (high aldehyde oxidase activity), at the dose of 50 mg/kg, 7-OH-MTX was detected in the bile as a major metabolite, together with MTX. However, 7-OH-MTX was detected in only small amounts in the bile of WKA/Sea strain, a low aldehyde oxidase activity strain, after MTX dosing.

**Strain difference of biliary excretion of 7-OH-MTX after MTX administration:** When biliary excretion of 7-OH-MTX and MTX was compared among the four strains of bile-drained rats, the cumulative excretion of 7-OH-MTX showed marked strain differences, as had been observed in MTX hydroxylase activity in vitro. The highest amount of 7-OH-MTX (6.2% of dose) was excreted into the bile of Sea:SD rats (up to 5 hr), and followed by Jcl:SD (3.2%) and Crj:SD (0.7%) rats. The lowest amount (0.02%) was observed in WKA/Sea rats. MTX was excreted into the bile in much higher amount than that of 7-OH-MTX, and the amounts of MTX in the bile of WKA/Sea strain was higher than that in Sea:SD rats. Both MTX and 7-OH-MTX were rapidly excreted into the bile, and cumulative excretion reached a plateau at about 3 hr after dosing (Fig. 3). These results suggest that the degree of 7-hydroxylation of MTX in rats in vivo closely reflects the strain differences of MTX 7-hydroxylase activity in the liver.

**Strain difference of urinary and fecal excretion of 7-OH-MTX after MTX administration:** The amounts of 7-OH-MTX excreted into the urine and feces of rats for 24 hr after MTX administration were compared among the four strains. The strain differences in urinary and fecal excretion of 7-OH-MTX showed similar patterns to those seen in bile. However, the excreted amounts of MTX into the feces were lower that those of the bile. This may be due to the enterohepatic circulation of MTX in rats. The highest amount of 7-OH-MTX was excreted into the feces of Sea:SD strain rats, followed by Jcl:SD, Crj:SD and WKA/Sea rats in that order. In contrast, 7-OH-MTX was excreted into the urine in relatively small amounts, but the pattern of strain difference remained the same (Table 1). Little excretion of MTX and 7-OH-MTX was observed after 24 hr.

**In vivo-in vitro relationship of MTX metabolism by aldehyde oxidase:** We found strain differences of recovery of 7-OH-MTX in the excreta of rats dosed MTX. The relative amounts of 7-OH-MTX excreted in the bile after MTX administration were well correlated with in vitro MTX 7-hydroxylase and benzaldehyde oxidase activities based on aldehyde oxidase among the four strains (Fig. 4). These results indicate that the strain differences of 7-OH-MTX formation from MTX in rats in vivo can be attributed primarily to differences

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**Table 1. Excretion of methotrexate (MTX) and 7-hydroxymethotrexate (7-OH-MTX) into urine and feces of rats dosed with MTX**

<table>
<thead>
<tr>
<th>Strain</th>
<th>7-OH-MTX % of dose</th>
<th>MTX % of dose</th>
<th>7-OH-MTX % of dose</th>
<th>MTX % of dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sea:SD</td>
<td>4.17 ± 0.87</td>
<td>11.8 ± 5.6</td>
<td>0.814 ± 0.187</td>
<td>18.1 ± 4.8</td>
</tr>
<tr>
<td>Jcl:SD</td>
<td>3.78 ± 1.50</td>
<td>15.3 ± 5.4</td>
<td>0.363 ± 0.112</td>
<td>26.1 ± 3.8</td>
</tr>
<tr>
<td>Crj:SD</td>
<td>0.890 ± 0.100</td>
<td>19.0 ± 1.1</td>
<td>0.017 ± 0.008</td>
<td>19.9 ± 2.0</td>
</tr>
<tr>
<td>WKA/Sea</td>
<td>0.165 ± 0.130</td>
<td>23.2 ± 3.8</td>
<td>0.003 ± 0.003</td>
<td>25.9 ± 0.8</td>
</tr>
</tbody>
</table>

The each value represents the mean ± SD of three rats. Amounts of MTX and 7-OH-MTX excreted into the urine and feces of rats for 24 hr were determined by HPLC after solid-phase extraction. Other details are described in Materials and Methods.
of aldehyde oxidase activity in the liver in vitro, and aldehyde oxidase functions as the major enzyme catalyzing this reaction.

Discussion

In this study, we examined strain differences in the conversion of MTX to 7-OH-MTX in rats in vivo and in vitro. Marked variability was found in liver cytosols from four strains of rats, and the difference between the highest (Sea:SD) and lowest (WKA/Sea) activities was 46-fold (Fig. 2A). Significant strain differences were also observed when the aldehyde oxidase activity of the four strains of rats was assayed in terms of benzaldehyde oxidase activity (Fig. 4). We have already reported marked strain differences of aldehyde oxidase activity in rats in an assay using benzaldehyde as a substrate: the highest activity was also observed with Sea:SD rats and the lowest with WKA/Sea rats among the strains examined, and the difference between the highest and lowest activities was 63.5-fold.20) It was suggested that quantitative, but not qualitative, differences of aldehyde oxidase exist among different strains of rats, since there was little variation in the observed $K_m$ values of the enzyme among the strains of rats examined, and Western blot analysis showed that the strains possess different quantities of the enzyme. No evidence was found to suggest the involvement of an inhibitor or stimulator as a causal agent of the strain differences in the enzyme activity. Strain difference of MTX-hydroxylase activity was also noted in our previous report.19) In the current study, we found strain differences of recovery of 7-OH-MTX in the excreta of rats dosed MTX. The relative amounts of 7-OH-MTX excreted in urine, feces and bile after MTX administration were well correlated with in vitro MTX 7-hydroxylase and benzaldehyde oxidase activities based on aldehyde oxidase among the four strains. Recovery of MTX excreted in feces and bile of rats dosed MTX was high in Crj:SD and WKA/Sea strain rats, but low in Sea:SD and Jcl:SD rats. This may be due to differences in the activities for formation of 7-OH-MTX and its glutamate in the liver in these strains. In contrast, MTX hydroxylase activity was not correlated with xanthine oxidoreductase activity (data not shown). These results indicate that the strain differences of 7-OH-MTX formation from MTX in rats in vivo can be attributed primarily to differences of aldehyde oxidase activity.

Aldehyde oxidase contains FAD, molybdenum and iron-sulfur centers, and can catalyze the oxidation of many aldehydes and nitrogenous heterocycles. 17) However, marked species differences and strain differences of the enzyme activity exist in mice and rats.17,20,26) Johns et al.7) reported that in rabbits, the hydroxylation of MTX is due to aldehyde oxidase, based on studies using the partially purified enzyme, while the metabolizing capacity of rat liver aldehyde oxidase is very low (about 0.5%) compared with that of the rabbit liver enzyme. In contrast, an investigation in isolated perfused rat liver showed that MTX passed through the liver without being metabolized.27) Rhee and Galivan28) demonstrated that MTX was converted to 7-OH-MTX in cultured rat hepatic cells, but the enzyme responsible could not be identified as aldehyde oxidase. In another study, aldehyde oxidase did not predominantly function as an MTX hydroxylase in rats.23) We found that aldehyde oxidase plays a major role in the hydroxylation of MTX in rat liver.19) In in vivo experiments, it was demonstrated that 7-OH-MTX was excreted into bile of rats dosed with MTX as the major route, indicating that the liver is a major site of 7-OH-MTX formation in the rat.9,29) Slordal et al.30) also reported that 7-OH-MTX was present in the blood of rats dosed with MTX, and the metabolite was eliminated with a longer half life than that of MTX. However, some authors have found that hydroxylation of MTX does not occur in rats in vivo.22,31) The discrepancies among

Fig. 4. Correlation of recovery of 7-hydroxymethotrexate (7-OH-MTX) in rat bile with in vitro liver aldehyde oxidase activity.
Aldehyde oxidase activities toward methotrexate (MTX) (A) and benzaldehyde (B) in liver cytosol of four rat strains were measured as described in Materials and Methods. Each value represents the mean ± SD of four rats. Excreted amounts of 7-OH-MTX in the bile are taken from Fig. 3.
the studies might again be due to strain differences of the MTX hydroxylase activity. When rats with a low aldehyde oxidase activity are used for the study of the metabolism of MTX, little MTX hydroxylation will be observed. Here, we show that 7-OH-MTX was mainly excreted into bile, together with MTX, and that the oxidation of MTX appeared to be catalyzed primarily by aldehyde oxidase. Xanthine oxidase is also reported to function as an MTX hydroxylase.21 However, we found a close correlation of MTX with aldehyde oxidase among four rat strains, while there appeared to be no relationship with xanthine oxidase activity. Therefore, we consider that xanthine oxidase contributes little to the 7-hydroxylation of MTX in vivo, at least in the strains of rats studied here.

When MTX is used clinically, the maintenance of an effective level is an important consideration. 7-OH-MTX is cytotoxic and also shows some pharmacological effects. Recently, inhibitory effects of some medicinal drugs, such as raloxifene, on human liver aldehyde oxidase effects. Recently, inhibitory effects of some medicinal drugs, such as raloxifene, on human liver aldehyde oxidase have been reported.32,33 Clinically, combined usage of MTX with cyclosporin resulted in a decrease in the level of 7-OH-MTX in blood of patients.34 Therefore, inherently low activity or external inhibition of MTX hydroxylation by aldehyde oxidase could be important in the clinical application of MTX. Variation of aldehyde oxidase activity in humans has been discussed in several papers,35,36,37 and individual variation in MTX hydroxylase activity in humans should be taken into consideration for clinical application.

References


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