Conversion of Methotrexate to 7-Hydroxymethotrexate and 7-Hydroxymethotrexate Polyglutamates in Cultured Rat Hepatic Cells

Myung S. Rhee and John Galivan

ABSTRACT

The formation of 7-hydroxymethotrexate and its diglutamate has been established in rat hepatic parenchymal cells in culture. The formation of 7-hydroxymethotrexate increases with the extracellular methotrexate concentration between 50 and 500 μM and remains constant over a 24-h period. The majority of the 7-hydroxy derivative is found in the medium after 6- and 24-h incubations at all concentrations examined. At high methotrexate concentration (50 μM) 7% of the total extracellular methotrexate was the 7-hydroxy derivative. 7-Hydroxymethotrexate diglutamate is accumulated within the cell, although longer chain length derivatives are not observed. The inability to form longer chain length polyglutamate derivatives is consistent with a limited capacity of hepatocytes to convert radiolabeled 7-hydroxymethotrexate to the tri- and tetraglutamates although the diglutamate is readily formed. A hepatoma cell line has an extremely limited capacity to form 7-hydroxy derivatives from methotrexate but form measurable amounts of mono-, di-, and triglutamates of 7-hydroxymethotrexate. 7-Hydroxymethotrexate was a good substrate for glutamyltylation in the hepatoma cells with large amounts of tri- through pentaglutamate derivatives. These studies confirm that 7-hydroxymethotrexate and the diglutamate must be considered when evaluating methotrexate pharmacology and demonstrate that this will be necessary with regard to methotrexate hepatotoxicity.

INTRODUCTION

The metabolism of MTX2 by rat hepatic parenchymal cells has been studied in some detail (1–8). These investigations have shown that the conversion of MTX to their poly(γ-glutamyl) derivatives occurs readily in hepatic parenchymal cells and that the resulting products are predominated by those containing a total of 2 through 4 γ-glutamate residues.

In these studies the formation of 7-OH-MTX was not noted. Clear evidence for the hydroxylation of MTX by aldehyde oxidase (EC 1.2.3.1) in extracts of rat liver has been presented (9), which suggests that hydroxylation may occur within hepatic parenchymal cells. Moreover the capacity of rat liver polyglutamate synthetase (EC 6.6.3.2.17) to catalyze the γ-glutamylation of 7-OH-MTX (10) suggests that 7-OH-MTX polyglutamates may also be formed in rat hepatocytes.

Because of the possibility of the hydroxylation reaction in rat liver we sought to determine if 7-OH-MTX and its polyglutamate derivatives were formed from MTX in vitro by rat hepatocytes and other liver derived cells. The present report is the result of these studies in which we also compare the relative capacity of hepatic parenchymal cells, nonparenchymal cells, and hepatoma cells to catalyze 7-hydroxylation of MTX and the γ-glutamylation of 7-OH-MTX.

MATERIALS AND METHODS

Materials. Folate-free Swim’s medium, LI5 medium, FBS, and horse serum were obtained from Grand Island Biological Co. (Grand Island, NY). Methotrexate was provided by Lederle Laboratories (Pearl River, NY), [3',5',7'-3H]MTX was purchased from Moravek Biochemicals (Brea, CA), and [γ-glutamyl-3,4-3H]methotrexate was purchased from New England Nuclear (Boston, MA). All were purified by DEAE-cellulose column chromatography prior to use (11). The specific activity of [3',5',7'-3H]MTX in these experiments was in the range of 1–2 × 10^6 dpm/nmol for parenchymal cells and 1–2 × 10^5 dpm/nmol for Kupffer cells. [γ-glutamyl-3,4-3H]-7-OH-MTX (specific activity, 2 × 10^5 dpm/nmol) was synthesized with rabbit liver aldheyde oxidase (10) and purified by DEAE-cellulose chromatography (11). Methotrexate polyglutamate standards (Glu1 through Glu5) were provided by the National Cancer Institute. 7-OH-MTX and rabbit liver aldehyde oxidase were the generous gift of Dr. J. J. McGuire of Yale University and Drs. Paul Newton and Raymond Blakley of St. Jude’s Children’s Hospital kindly provided the 7-OH-MTX polyglutamate standards. DEAE-cellulose was purchased from Scheicher and Schuell (Keene, NH). Collagenase (type I), DNase (type I), metrizamide, dexamethasone, insulin, and DL-α-tocopherol were purchased from Sigma Chemical Co. (St. Louis, MO), Pronase (Type I) was from Calbiochem (La Jolla, CA), ethylene glycol bis(β-aminoethylether)-N,N,N’,N’-tetraacetic acid was from Lamont Lab (Dallas, TX), Aquasol was from New England Nuclear (Boston, MA), and Beckman Ready-Solv was from Beckman Instruments (Irvine, CA).

Hepatocyte Isolation and Culture. Male Lewis rats (220–350 g) were supplied by Charles River Breeding Laboratories. They were kept for at least 2 weeks for adaptation on a 12-h inverted light-dark cycle. At the end of the light period the hepatocytes were isolated as described previously (5, 8) with 0.05% collagenase perfusion. Hepatic parenchymal cells were prepared by low-speed centrifugation and plated at a density of 2.5 × 10^6 cells in 4 ml L15 medium containing 0.1 μM insulin and 15% FBS on a 60-mm Falcon plastic dish coated with collagen (100 μg/dish). Attachment of the cells to the dish occurred within 3 to 4 h at 37°C and the medium was then changed to folate-free Swim’s medium supplemented with 0.1 μM insulin, 0.1 μM dexamethasone, and 6 μM α-tocopherol. The cells were generally utilized for experiments after they had been in culture for 20 h unless otherwise noted. Kupffer cells were isolated according to the method of Munthe-Kaas et al. (12) with modification. Briefly, the initial hepatocyte suspension prepared by collagenase perfusion was further treated with 0.2% Pronase and 0.1 mg DNase at 37°C for 1 h, which destroyed the parenchymal cells. Nonparenchymal cells were further purified from cell debris and residual RBC by being layered on metrizamide suspension (density, 1.08 g).

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The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. 1Recipient of NIH Grants CA25933 and CA34314 by the National Cancer Institute, USPHS, Department of Health and Human Service. To whom requests for reprints should be addressed. 2Abbreviations used: MTX (4-NH2-10-CH3PteGlu), methotrexate, 4-amino-10-methylpteroylglutamate; HPLC, high performance liquid chromatography; FBS, fetal bovine serum; 7-OH-MTX (4-NH2-7-OH-10-CH3PteGlu), 7-hydroxymethotrexate. The polyglutamate chain length of MTX and 7-OH-MTX is indicated by a subscript in the abbreviation; e.g., 4-NH2-10-CH3PteGlu or 4-NH2-7-OH-10-CH3PteGlu, each contain one additional glutamate γ-linked to MTX or 7-OH-MTX, respectively.
tion of [3H]MTX in folate-free Swim’s medium with hormone supplement which in the case of H35 cells included only insulin. Total [3H]MTX in the cells was determined as described previously (11). In all cases the intracellular composition of MTX and its metabolites was determined as the mean of duplicate samples and expressed as nmol product/g cell protein (5, 6). The distribution of MTX and its 7-hydroxy and poly(γ-glutamyl) metabolites was determined by subjecting boiled H2O extracts of the cells to a modified HPLC system used previously to resolve MTX and polyglutamates (13). In the present study the octadecylsyl column was washed isocratically for 10 min with 4% acetonitrile and then with a linear gradient of 4 to 11% acetonitrile for 40 min. The MTX and 7-OH-MTX from the incubation medium were resolved by this method and also by the method of Newton and Blakley (14). When the conversion of [3',5',7-3H]MTX to [3',5',7-3H]-7-OH-MTX and [3',5',7-3H]-7-OH-MTX polyglutamates was measured a correction of 60% was made due to the loss of 3H from position 7. The amount lost was determined by treatment of [3',5',7-3H]MTX with aldehyde oxidase until complete conversion to [3',5',7-3H]7-OH-MTX occurred (10).

RESULTS

Resolution of 7-OH-MTX Derivatives. Previous studies on the metabolism of MTX in hepatocytes and other tissues have used numerous chromatographic procedures to resolve methotrexate from the polyglutamate derivatives. Those that have been utilized in this laboratory [gel filtration (11), DEAE-cellulose chromatography (6), and reversed phase HPLC utilizing a C18 column with an acetonitrile gradient (13)] are not capable of simultaneous resolution and identification of 7-OH-MTX and MTX and all of the possible polyglutamate derivatives. Therefore we used a modification of the previous HPLC system (13) and the system developed by Newton and Blakley (14) to allow resolution of the metabolites of MTX observed in hepatocytes (Fig. 1).

Time Dependence of 7-OH-MTX Formation. The appearance of the 7-hydroxy derivatives of MTX was examined as a function of time at an extracellular concentration of 25 μM MTX (Fig. 2). This is the concentration at which the rate of glutamyl attraction of MTX reaches saturation (8). Modest amounts of intracellular 7-OH-MTX are observed in the first two h which accumulate to the extent of 28% of the intracellular MTX by 6 h. Polyglutamate derivatives of MTX and 7-OH-MTX are also synthesized during the incubation. At 6 h 4-NH2-7-OH-CH3PteGlu2 is the major MTX polyglutamate (approximately 70%) with lesser amounts of the Glu3 and Glu4 derivatives (8). 4-NH2-7-OH-10-CH3PteGlu2 was the only polyglutamate species of 7-OH-MTX consistently observed (see below).

The appearance of 7-OH-MTX in the medium was observed even at the early time points, and the amounts were approximately the same as that in the cell for 2 h. At later times the extracellular concentration of 7-OH-MTX exceeded the intracellular concentration. Thus it appears that under the conditions used here there is a continuous synthesis of 7-OH-MTX which results in large amounts of compound being released into the medium. After a 6-h incubation with 25 μM MTX the extracellular 7-OH-MTX exceeds the intracellular by approximately nearly 3-fold. Thus the major product of the metabolism of MTX in rat hepatocytes over 6 h is the 7-hydroxy derivative followed by 4-NH2-7-OH-10-CH3PteGlu2 (8) and 4-NH2-7-OH-10-CH3PteGlu2. Lesser amounts of 4-NH2-10-CH3PteGlu2 and Glu4 are also formed (8).

Concentration Dependence of 7-OH-MTX Formation. The formation of 7-OH-MTX as a function of the concentration of MTX in the medium in hepatocytes when measured after 6 h incubation is demonstrated in Table 1. Under these conditions the cellular accumulation of unmetabolized MTX is not saturated, which is consistent with the low affinity transport system for MTX in rat hepatocytes which has been described by several investigators (1-5, 15). A notable characteristic of this process is the capacity of the hepatocytes to accumulate high levels of unmetabolized MTX. The formation of 7-OH-MTX increases directly with the amount of MTX associated with the cell and appears in the medium at the lowest MTX concentration examined. At 6 h the total amount of 7-OH-MTX (cells plus medium) is approximately equivalent to the intracellular MTX. With a longer incubation time further synthesis of 7-OH-MTX is observed and 7% of the medium MTX is converted to 7-OH-MTX when the hepatocytes are exposed to 50 μM MTX for 24 h. It should be noted that the reduced MTX in the cells at 24 h is due to the loss in the capacity of hepatocytes to convert MTX as a function of the time that they are in culture (5).

The diglutamate derivative of 7-OH-MTX (4-NH2-7-OH-10-CH3PteGlu2) is formed, but there appears to be no great in-
Table 1  Formation of 7-OH-MTX species as a function of external MTX concentration during 6 h incubation

Hepatocytes which had been cultured in folate free Swim's medium containing insulin, desmethylamethotrexate, and α-tocopherol for 24 h were incubated in the same medium for 6 h with the indicated concentrations (1–50 μM) of [3H]MTX. At 6 or 24 h cells were washed three times with cold phosphate buffered saline and cellular extracts were collected and analyzed as described in "Materials and Methods." The number of glutamates (GLU4) indicates the total number of glutamate residues in the product.

<table>
<thead>
<tr>
<th>Cellulose concentration (nmol/g protein) of 7-OH-MTX</th>
<th>Medium concentration (nmol/g protein) of 7-OH-MTX</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTX (μM)</td>
<td>GLU1</td>
</tr>
<tr>
<td>1</td>
<td>16.5 ± 1.0</td>
</tr>
<tr>
<td>2</td>
<td>32.3 ± 3.8</td>
</tr>
<tr>
<td>5</td>
<td>80.5 ± 9.2</td>
</tr>
<tr>
<td>10</td>
<td>234 ± 21</td>
</tr>
<tr>
<td>25</td>
<td>576 ± 46</td>
</tr>
<tr>
<td>50</td>
<td>1091 ± 99</td>
</tr>
<tr>
<td>50*</td>
<td>309 ± 27</td>
</tr>
</tbody>
</table>

* Mean ± SE (n = 3). Data for each experiment were averages of duplicates in three different preparations of hepatocytes.
* The results are expressed as the mean of two independent experiments.
* Incubation with MTX was extended to 24 h.

Table 2  Formation of 7-OH-MTX species in hepatoma cells

Confluent H353 cells which had been maintained in folate free Swim's medium with 0.1 μM insulin for 24 h were incubated with 10 μM [3H]MTX for 6 h.

<table>
<thead>
<tr>
<th>Extracellular MTX (μM)</th>
<th>Cellular concentration (nmol/g) of 7-OH-MTX</th>
<th>Medium (nmol/g) of 7-OH-MTX</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GLU1</td>
<td>GLU2</td>
</tr>
<tr>
<td>10</td>
<td>0.85* (10)</td>
<td>3.6</td>
</tr>
</tbody>
</table>

* The results are the mean of two independent experiments.
* Numbers in parentheses, amount of corresponding MTX polyglutamate in the cells formed during the incubation. The total amount of methotrexate within the cells including 7-hydroxy derivative and polyglutamates is 180.7 nmol/g.

In contrast the hepatoma cells readily generated large amounts of the tri- through pentaglutamate derivatives with 7-OH-MTX as the substrate (Table 3). The distribution among the various polyglutamates is generally similar to MTX, but the total accumulation is approximately 70% that of the MTX polyglutamates (13). These results demonstrate that the hepatoma cells can readily form 7-OH-MTX polyglutamate when presented with appropriate substrate, but their capacity to generate 7-hydroxy derivatives from MTX is reduced especially in comparison to normal hepatocytes.

DISCUSSION

The present study demonstrates that under a variety of conditions 7-OH-MTX is the major metabolite of methotrexate in cultured cells with regard to 7-hydroxy and polyglutamate derivatives.

Analyses of the amount of glutamyl and hydroxylation were also made in the H353 hepatoma cell line (11). During a 6-h incubation with 10 μM MTX, 10.6 nmol of the MTX pool/g cell protein are hydroxylated (Table 2). Small amounts of the 7-hydroxy derivatives of 4-NH2-10-CH3PteGlU2 and GLU4 are found in the H353 cells and limited amounts of 7-OH-MTX are detected in the medium. The total 7-hydroxy species formed by the hepatoma cells are 5.5% of the MTX and MTX polyglutamate pool. Thus, the transformed hepatic cell, which is more active in glutamylating methotrexate than hepatocytes (8, 13), has a greatly reduced capacity for 7-hydroxylation.

Glutamylation of 7-OH-MTX by Hepatocytes. Both hepatocytes and hepatoma cells exhibit a different but limited capacity to form polyglutamate derivatives of 7-OH-MTX when incubated with MTX. We therefore evaluated the ability of 7-OH-MTX to act as a substrate for glutamylations in these two cell lines (Table 3). The hepatocytes were incubated with 2 μM 7-OH-MTX for 6 and 24 h. This concentration of MTX favors the formation of longer chain length MTX polyglutamates (8). While the diglutamate was readily formed there was a very limited formation of longer chain length derivatives of 7-OH-MTX. With MTX as the substrate much greater amounts of 2-NH2-4-OH-PteGlU3 and GLU4 are formed and at the later time these two species are the chief polyglutamate derivatives (7, 8). The results indicate that the lack of longer chain length polyglutamate derivatives of 7-OH-MTX when MTX is the substrate may be related to the reduced capacity to glutamate the di- and triglutamate derivatives of 7-OH-MTX. This combined with the reduced ability of aldehyde oxidase to hydroxylate MTX polyglutamates relative to MTX (10) would result in restricted formation of the longer chain length 7-OH-MTX polyglutamates with MTX as the substrate.

In conclusion the hepatoma cells readily generated large amounts of the tri- through pentaglutamate derivatives with 7-OH-MTX as the substrate (Table 3). The distribution among the various polyglutamates is generally similar to MTX, but the total accumulation is approximately 70% that of the MTX polyglutamates (13). These results demonstrate that the hepatoma cells can readily form 7-OH-MTX polyglutamate when presented with appropriate substrate, but their capacity to generate 7-hydroxy derivatives from MTX is reduced especially in comparison to normal hepatocytes.

Table 3  Metabolism of 7-OH-MTX by hepatocytes

Hepatocytes were cultured in folate free Swim's medium containing insulin, desmethylamethotrexate, and α-tocopherol for 24 h were incubated with 2 μM (L-glutamyl-3,4-3H)7-OH-MTX for 6 and 24 h. Confluent H353 hepatoma cells were treated the same way except that the medium was folate-free Swim's medium with 0.1 μM insulin.

<table>
<thead>
<tr>
<th>Incubation</th>
<th>Cellular concentration (nmol/g protein) of 7-OH-MTX</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GLU1</td>
</tr>
<tr>
<td>Hepatocytes*</td>
<td>6</td>
</tr>
<tr>
<td>24</td>
<td>9.1 ± 0.7</td>
</tr>
<tr>
<td>Hepatoma cells*</td>
<td>6</td>
</tr>
<tr>
<td>24</td>
<td>21.0</td>
</tr>
</tbody>
</table>

* Mean ± SE (n = 4).
* Average of two experiments.

3 J. Galivan, unpublished results.
cultured rat hepatocytes. Precedent for this possibility arises from the identification of aldehyde oxidase in rat liver (9). Furthermore the synthesis of polyglutamate derivatives of 7-OH-MTX was suggested by studies showing that polyglutamate synthetase could catalyze the glutamylation of 7-OH-MTX (10, 16) and that aldehyde oxidase is active on MTX polyglutamates albeit less than on MTX (10, 17). Previous investigations have not detected 7-OH-MTX in cultured rat hepatocytes (1–7) although a more recent, detailed analysis of MTX polyglutamate formation noted 4-NH$_2$-7-OH-10-CH$_3$PteGlu$_2$ and accounted for this metabolite (8). The reasons for the difficulty in detecting the 7-hydroxy derivatives have been suggested by McGuire et al. (10). The majority of the 7-OH-MTX is lost to the medium by the hepatocytes under most of the conditions utilized in the present study (Table 1). The presence of the 7-hydroxy derivatives is further obscured by the fact that 60% of the tritium is lost during the hydroxylation procedure. Furthermore, care must be taken to ensure that resolution of MTX, MTX polyglutamates, and the 7-hydroxy derivatives can be achieved.

While the conversion of MTX to 7-OH-MTX has been described in several systems in vivo including that of humans (18–22) and with liver extracts from various species (10, 17, 23, 24), a limited number of studies have been conducted with cultured cells (14, 25). The in vitro studies have demonstrated a very rapid and nearly quantitative conversion of MTX to 7-OH-MTX in rabbit hepatocytes (25), which is probably the reason for the reduced toxicity of MTX to rabbits (26). CCRF-CEM cells catalyze the formation of 7-OH-MTX when incubated with 100 µM MTX. Significant amounts of MTX polyglutamates were formed during the incubation but 7-OH-MTX polyglutamates were not detected (14).

Although 7-OH-MTX is the major pathway of MTX metabolism in rat hepatocytes, the formation of MTX polyglutamates (4-NH$_2$-7-OH-10-CH$_3$PteGlu$_2$-Glu$_2$) (7, 8) and 4-NH$_2$-7-OH-10-CH$_3$PteGlu$_2$ (Table 1) is also observed. To our knowledge these studies are the first observation of the conversion of MTX to 7-OH-MTX polyglutamates in a cell culture system although this possibility has been postulated (10, 17). Longer glutamyl chain length derivatives of 7-OH-MTX were not observed in hepatocytes with MTX as substrate under the experimental conditions examined here. This is consistent with the limited ability of hepatocytes to form longer chain length polyglutamates with 7-OH-MTX as substrate (Table 3).

The hepatoma cells utilized in this study also form 7-OH-MTX and polyglutamates but the amounts are quite small. These cells have approximately 5-fold the glutamylation capacity of hepatocytes (8, 13) and are highly active in converting 7-OH-MTX to polyglutamates with 2 through 5 glutamate residues. The limited hydroxylation that occurs may be due to low levels of aldehyde oxidase. In addition the hepatoma cells accumulate only limited amounts of unmetabolized MTX. The high $K_m$ of aldehyde oxidase for MTX (24) in addition to its high $K_m$ of aldehyde oxidase for MTX (24) in addition to its reduced activity on MTX polyglutamates may also contribute to the limited conversion of MTX to 7-OH-MTX in H35 hepatoma cells. Greater amounts of 7-OH-MTX could conceivably compromise the toxicity of MTX to the tumor cells (27).

The formation of 7-OH-MTX polyglutamates from MTX can be catalyzed either by the hydroxylation of MTX polyglutamates (10, 17) or by the glutamylation of 7-OH-MTX (10, 16, 17, 28, 29) and it is not clear from the present studies which of these is the primary pathway. The facts that the concentration of 4-NH$_2$-7-OH-10-CH$_3$PteGlu$_2$ (Fig. 1; Table 1) is nearly equal to that of 4-NH$_2$-10-CH$_3$PteGlu$_2$ and that the diglutamate of MTX is a relatively poor substrate for aldehyde oxidase suggest that the diglutamate derivative of 7-OH-MTX may be derived primarily from the glutamylation of 7-OH-MTX. This would be consistent with the good substrate activity of 7-OH-MTX for glutamylation (Table 3; Refs. 10, 16, 17, 28 and 29). However, both pathways may contribute to the formation of 4-NH$_2$-7-OH-10-CH$_3$PteGlu$_2$. The substrate activity of MTX and 7-OH-MTX for glutamylation varies in different biological systems. In certain cases the 7-hydroxy derivative is a better substrate (17, 28), while in others it is nearly equivalent (10) or poorer (16). In the cell systems utilized here it appears somewhat less efficient.

The role of 7-OH-MTX in the cytotoxicity of methotrexate is presently unclear. Large amounts of the material are produced especially with high dose methotrexate therapy. A consensus of data suggests that the 7-hydroxy derivatives may limit MTX toxicity (27–30) to dividing cells by interfering with MTX uptake (30) and polyglutamylation (10, 16, 17).

The presence of 4-NH$_2$-7-OH-10-CH$_3$PteGlu$_2$, and Glu$_2$ in hepatocytes and their role in the hepatotoxicity caused by MTX is unclear especially since there are several aspects of MTX toxicity at this site. MTX dependent hepatotoxicity is a complication only with long term, low dose pulse exposure to the drug (31, 32). The liver normally consists of nondividing cells which are in G$_0$ (33). Thus very little de novo thymidylate biosynthesis is required resulting in a reduced formation of dihydrofolate (34). Hence the inhibition of the target enzyme dihydrofolate reductase (EC 1.5.1.3) may play a limited role in MTX hepatotoxicity. Alternative molecular site(s) of hepatotoxicity have been proposed (35) but a clear understanding of this problem is not yet available. It does seem that the polyglutamate derivatives of MTX are forms which are likely to be causing this pathology because of their enhanced hepatocyte retention (1–4, 7, 8). Rat liver parenchymal cells are of interest with regard to human liver in that both species develop low dose long term methotrexate hepatotoxicity (36). The finding of polyglutamate derivatives of 7-OH-MTX derived from MTX in hepatocytes requires that these molecules be considered in evaluating MTX-induced hepatotoxicity.

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REFERENCES

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7-HYDROXYMETHOTREXATE IN RAT HEPATIC CELLS


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