Reactions of Methotrexate with Hypobromous Acid and Hypochlorous Acid

Toshinori Suzuki* and Rie Takeuchi

School of Pharmacy, Shujitsu University; 1–6–1 Nishigawara, Okayama 703–8516, Japan.

Received July 19, 2019; accepted August 20, 2019

Introduction

Methotrexate (MTX), also known as amethopterin, is one of the oldest drugs but is still frequently used.1–3) MTX is structurally similar to folic acid and acts as an antimetabolite, interfering with the metabolism of folic acid. MTX competitively inhibits dihydrofolate reductase synthesizing tetrahydrofolate that provides single carbon groups for the synthesis of precursors of DNA and RNA.4) Thus, MTX can inhibit cell proliferation. High doses of MTX are prescribed for the treatment of cancer,5) while low doses MTX are widely prescribed for the treatment of rheumatoid arthritis.6,7) These diseases treated with MTX involve inflammation. At sites of inflammation, eosinophils and neutrophils are recruited. Eosinophils, one of the white blood cell types, are abundant in blood and tissues in various inflammatory disorders.8) Eosinophil peroxidase, secreted by eosinophils, generates hypobromous acid (HOBr) using H2O2 and Br−.9,10) Although the plasma concentrations are approx. 60 µM for Br− and 100 mM for Cl−, eosinophil peroxidase uses Br− preferentially to generate HOBr.11,12) Meanwhile, neutrophils are a major component of white blood cells. Myeloperoxidase, secreted by neutrophils, generates hypochlorous acid (HOCI) from H2O2 and Cl−13–15) which plays a central role in host defense mechanisms against infection. HOCI can oxidize Br− to generate HOBr.16,17) Thus, a portion of the HOCl formed by the myeloperoxidase system in humans should react with Br−, converting to HOBr. Under inflammation conditions, MTX may encounter HOBr and HOCl, and react with them. In the present study, we examined the reactions of MTX with HOBr and HOCl, and report that both HOBr and HOCl react with MTX to generate monohalogenated products. To the best of our knowledge, this is the first study to examine the reactions of MTX with HOBr and HOCl under neutral conditions, although the reactions of MTX with Br2 and Cl2 in hydrochloric acid were reported.18

Key words: methotrexate; hypobromous acid; hypochlorous acid; 3′-bromomethotrexate; 3′-chloromethotrexate

Results

Reaction with HOBr

A solution of 100 µM MTX was incubated with 100 µM HOBr in 100 mM potassium phosphate buffer (pH 7.4) at 37°C for 30 min. When the reaction mixture was analyzed by reversed-phase (RP) HPLC, a product peak appeared in the chromatogram detected at 260 nm (Fig. 1). The product peak showed a UV spectrum ($\lambda_{\text{max}} = 260$ and 372 nm) as shown in the inset of Fig. 1. The product was isolated by RP-HPLC and identified using MS (electrospray ionization time of flight mass spectrometry, ESI-TOF/MS) and NMR (1H-1d, 1H–1H correlation spectroscopy (COSY), 1H–13C heteronuclear multiple quantum correlation (HMQC), 1H–13C heteronuclear multiple bond correlation (HMBC), 13C-1d). The product showed an ESI-TOF/MS spectrum including peaks of $m/z = 531$ and 533 (1:1) in negative mode. High-resolution (HR) ESI-TOF/MS values of the molecular ion ($m/z = 531$) for the product agreed with the theoretical molecular mass for C20H21BrN8O5 attributed to [MTX + Br⁻ + 2H⁺] within 3 ppm. A singlet aromatic 1H signal attributable to the 7-position of the pteridine moiety was observed. For the benzoyl group, three aromatic protons were observed. Two of them, attributable to 2′ and 6′ protons, showed correlations with the carbonyl carbon on the 1H–13C HMBC spectrum. From these data, the product was identified as 3′-bromomethotrexate (3′-Br-MTX) (Fig. 2).

Figure 3A shows the time-dependence of the reaction of MTX with HOBr when 100 µM MTX and 100 µM HOBr were incubated in 100 mM potassium phosphate buffer at pH 7.4 and 37°C for 0–4 h. The reaction was fast and completed within 10 min. Figure 3B shows the HOBr dose-dependence of the reaction of MTX with HOBr when 100 µM MTX and 0–100 µM HOBr were incubated in 100 mM potassium phosphate buffer at pH 7.4 and 37°C for 30 min. With increasing HOBr concentration, the consumption of MTX and the formation of 3′-Br-MTX increased. MTX was converted to 3′-Br-MTX almost exclusively.

© 2019 The Pharmaceutical Society of Japan
Reaction with HOCl
A solution of 100 \( \mu M \) MTX was incubated with 100 \( \mu M \) HOCl in 100 mM potassium phosphate buffer (pH 7.4) at 37°C for 30 min. A product peak appeared in the RP-HPLC chromatogram detected at 260 nm (Fig. 4). The peak showed a UV spectrum (\( \lambda_{max} = 261 \) and 372 nm) similar to 3'-Br-MTX. The product was isolated by RP-HPLC and identified using MS and NMR. The product showed a ESI-TOF/MS spectrum including peaks of \( m/z = 487 \) and 489 (3 : 1) in negative mode. HR-ESI-TOF/MS values of the molecular ion (\( m/z = 487 \)) for the product agreed with the theoretical molecular mass for \( \text{C}_{20}\text{H}_{21}\text{ClN}_{8}\text{O}_{5} \) attributable to \([\text{MTX} + \text{Cl}−\text{2H}]^-\) within 3 ppm. For NMR, results similar to those of 3'-Br-MTX were obtained. A singlet aromatic 1H signal attributable to the 7-position of the pteridine moiety was observed. For the benzoyl group, three aromatic protons were observed. Two of them, attributable to 2' and 6' protons, showed correlations with the carbonyl carbon on the 1H–13C HMBC spectrum. From these data, the product was identified as 3'-chloromethotrexate (3'-Cl-MTX) (Fig. 5).

Figure 6A shows the time-dependence of the reaction of MTX with HOCl when 100 \( \mu M \) MTX and 100 \( \mu M \) HOCl were incubated in 100 mM potassium phosphate buffer (pH 7.4) at 37°C for 0–4 h. The reaction was fast and completed within 10 min.

Reaction with HOCl in the Presence of NaBr
A solution of 100 \( \mu M \) MTX was incubated with 100 \( \mu M \) HOCl in the presence of 0–100 \( \mu M \) NaBr in 100 mM potassium phosphate buffer (pH 7.4) at 37°C for 30 min (Fig. 7). As the NaBr dose was increased up to 100 \( \mu M \), the concentration of 3'-Cl-MTX decreased, while the concentration of 3'-Br-MTX increased.

Effects of Additives on the Reaction with HOBr
To obtain information about the effect of coexistent compounds on the reactions of MTX with HOBr, experiments were carried out in the presence of various additives. Table 1 shows the concentrations of MTX and 3'-Br-MTX when a solution of 100 \( \mu M \) MTX and 1 mM additives was incubated with 100 \( \mu M \) HOBr in 100 mM potassium phosphate buffer (pH 7.4) at 37°C for 24 h. Ammonium chloride suppressed the reaction. Methylamine and dimethylamine showed little effect, while trimethylamine and tetramethylammonium showed no effect. Amino acids suppressed the reaction. Taurine and urea showed no effect.

Fig. 1. RP-HPLC Chromatogram of a Reaction Mixture of MTX with HOBr Detected at 260 nm
A solution of 100 \( \mu M \) MTX was incubated with 100 \( \mu M \) HOBr in 100 mM potassium phosphate buffer at pH 7.4 and 37°C for 30 min. The HPLC system consisted of LC-10ADvp pumps and an SPD-M10Avp UV-vis photodiode-array detector (Shimadzu). For the RP-HPLC, an Inertsil ODS-3 octadecylsilane column of 4.6 × 250 mm and particle size 5 \( \mu m \) (GL Sciences) was used. The eluent was 20 mM ammonium acetate (pH 7.0) containing methanol. The methanol concentration was increased from 0 to 50% during 15 min in linear gradient mode, and maintained at 50% from 15 to 30 min. The column temperature was 40°C and the flow rate was 1 mL/min.

Fig. 2. Structure of 3'-Bromomethotrexate (3'-Br-MTX)

Fig. 3. (A) Time Course of the Concentration Changes in MTX and 3'-Br-MTX When a Solution of 100 \( \mu M \) MTX Was Incubated with 100 \( \mu M \) HOBr in 100 mM Potassium Phosphate Buffer at pH 7.4 and 37°C for 0–4 h
(B) HOBr dose-dependence of the concentration changes in MTX and 3'-Br-MTX when a solution of 100 \( \mu M \) MTX was incubated with 0–100 \( \mu M \) HOBr in 100 mM potassium phosphate buffer at pH 7.4 and 37°C for 30 min. MTX (closed circle) and 3'-Br-MTX (open triangle). All the reaction mixtures were analyzed by RP-HPLC. Means ± standard deviation (S.D.) (n = 3) are presented.
effect. Ascorbic acid suppressed the reaction. Glucose and sodium acetate showed no effect.

Effects of Additives on the Reaction with HOCl

To obtain information about the effect of coexistent compounds on the reactions of MTX with HOCl, experiments were carried out in the presence of various additives. Table 2 shows the concentrations of MTX and 3'-Cl-MTX when a solution of 100 µM MTX and 1 mM additives was incubated with 100 µM HOCl in 100 mM potassium phosphate buffer (pH 7.4) at 37°C for 24 h. Ammonium chloride, methylamine, and dimethylamine strongly suppressed the reaction, while trimethylamine and tetramethylammonium showed little effect. Amino acids and taurine suppressed the reaction almost completely. Urea, glucose, and sodium acetate showed little suppression. Ascorbic acid suppressed the formation of 3'-Cl-MTX almost completely, although a certain amount of MTX was consumed.

Discussion

3'-Br-MTX and 3'-Cl-MTX were reportedly synthesized from MTX in hydrochloric acid by Br₂ and Cl₂, respectively. In the present study, we showed that MTX reacted with HOBr and HOCl to generate 3'-Br-MTX and 3'-Cl-MTX in a neutral solution, respectively. The reactions were fast and generated the corresponding halogenated MTX almost exclusively. Recently, we showed that rebamipide, a therapeutic agent for gastric ulcers and chronic gastritis, reacted with HOBr to yield several products including 3-bromorebamipide as a major product. Rebamipide did not react with HOCl. Under similar conditions, MTX reacted with not only HOBr but also HOCl to generate the corresponding monohalogenated products. Generally, the reactivity of HOBr to organic compounds is higher than that of HOCl due to the higher electrophilicity of HOBr compared to HOCl. MTX reacted with HOCl comparably to HOBr, especially at a low dose (0.25 mM) of the hypohalous acids as shown in Figs. 3B and 6B. This suggests that the reactivity of HOCl to MTX is relatively high. In the presence of NaBr, HOCl generated 3'-Br-MTX from MTX in addition to 3'-Cl-MTX as shown in Fig. 7. At around serum concentration of Br⁻ (approx. 60 µM), the formation of 3'-Br-MTX was comparable to that of 3'-Cl-MTX, suggesting that 3'-Br-MTX, in addition to 3'-Cl-MTX, may also generated in vivo by HOCl formed by myeloperoxidase.

CH₃NH₂ and (CH₃)₂NH had little effect on the reactions of MTX with HOBr, whereas they suppressed the reactions with HOCl almost completely (Tables 1, 2). CH₃NH₂ and (CH₃)₂NH react with HOBr and HOCl to generate corresponding bromamines and chloramines, respectively. It has been reported that second-order rate constants for the reactions of phenol with bromamines of CH₃NH₂ and (CH₃)₂NH are several orders higher than those with chloramines. Similarly, MTX would react with bromamines of CH₃NH₂ and (CH₃)₂NH to generate Fig. 6. (A) Time Course of the Concentration Changes in MTX and 3'-Cl-MTX When a Solution of 100 µM MTX Was Incubated with 100 µM HOBr in 100 mM Potassium Phosphate Buffer at pH 7.4 and 37°C for 0–4 h

(B) HOCl dose-dependence of the concentration changes in MTX and 3'-Cl-MTX when a solution of 100 µM MTX was incubated with 0–100 µM HOCl in 100 mM potassium phosphate buffer at pH 7.4 and 37°C for 30 min. MTX (closed circle) and 3'-Cl-MTX (open square). All the reaction mixtures were analyzed by RP-HPLC. Means ± S.D. (n = 3) are presented.
3'-Br-MTX, whereas MTX would not react with chloramines. The reactions of both HOBr and HOCl were suppressed by amino acids, even Gly. It has been reported that α-amino acids react with both HOBr and HOCl to yield corresponding aldehydes and nitriles via monobromamines and dibromamines of the α-amino acids, respectively. The reaction was suppressed more strongly by Lys, Met, and Cys than by Gly. This implies that, in addition to the reaction on the α-amino group, a γ-amino group for Lys, a methyl thiol group for Met, or a thiol group for Cys in the side chain reacts with HOBr and HOCl. The reaction of MTX with HOBr and HOCl in plasma should be suppressed by free amino acids, since the total concentration of free amino acids in human plasma is several mM. Taurine suppressed the HOCl reaction almost completely, whereas taurine did not suppress the HOBr reaction. Taurine reacts with HOBr and HOCl resulting in taurine bromamine (Tau-NHBr) and taurine choline (Tau-NHCl), respectively. It has been reported that the reactivity of Tau-NHBr to amino acids is higher than that of Tau-NHCl. Tau-NHBr may react with MTX to generate 3'-Br-MTX, whereas Tau-NHCl could not react with MTX. Although the taurine concentration is low in plasma (38 µM), it is high in neutrophils (19 mM) and eosinophils (15 mM). The reaction of MTX with HOCl generated by myeloperoxidase might be suppressed by taurine, whereas the reaction with HOBr by eosinophil peroxidase may not be suppressed.

Several studies have examined the concerning biological activities of 3'-Br-MTX and 3'-Cl-MTX. 3'-Br-MTX, as well as MTX, suppressed uptake of tritium-labeled MTX (MTX-3H) by the choroid plexus of a rabbit brain in vitro. Bromine-77-labeled 3'-Br-MTX (3'-77Br-MTX) injected into the femoral artery of a tumor bearing rat leg together with MTX showed high initial retention activity by the tumor. These reports indicate that 3'-Br-MTX can be taken up in organs or cells comparable to MTX. A Yoshida lymphosarcoma line resistant to an alkylating agent showed collateral sensitivity against 3'-Br-MTX. The original Yoshida sarcoma was transplanted into rats, which were then treated with 3'-Br-MTX. Initially, the tumor volume reduced, but tumor regrowth commenced, and less response was seen to the second dose. Some of the 3'-Br-MTX treated tumors were then transplanted into other rats. Treatment of the retransplanted tumors with the alkylating agent reduced tumor successfully. This suggests that 3'-Br-MTX can work as an antitumor reagent as well as or stronger than MTX under certain conditions. 3'-Cl-MTX inhibited both cultured human leukemic lymphoblasts and the corresponding MTX-resistant subline at a similar efficiency. The reactions of both HOBr and HOCl were suppressed by free amino acids, even Gly. It has been reported that α-amino acids react with both HOBr and HOCl to yield corresponding aldehydes and nitriles via monobromamines and dibromamines of the α-amino acids, respectively. The reaction was suppressed more strongly by Lys, Met, and Cys than by Gly. This implies that, in addition to the reaction on the α-amino group, a γ-amino group for Lys, a methyl thiol group for Met, or a thiol group for Cys in the side chain reacts with HOBr and HOCl. The reaction of MTX with HOBr and HOCl in plasma should be suppressed by free amino acids, since the total concentration of free amino acids in human plasma is several mM. Taurine suppressed the HOCl reaction almost completely, whereas taurine did not suppress the HOBr reaction. Taurine reacts with HOBr and HOCl resulting in taurine bromamine (Tau-NHBr) and taurine choline (Tau-NHCl), respectively. It has been reported that the reactivity of Tau-NHBr to amino acids is higher than that of Tau-NHCl. Tau-NHBr may react with MTX to generate 3'-Br-MTX, whereas Tau-NHCl could not react with MTX. Although the taurine concentration is low in plasma (38 µM), it is high in neutrophils (19 mM) and eosinophils (15 mM). The reaction of MTX with HOCl generated by myeloperoxidase might be suppressed by taurine, whereas the reaction with HOBr by eosinophil peroxidase may not be suppressed.

Several studies have examined the concerning biological activities of 3'-Br-MTX and 3'-Cl-MTX. 3'-Br-MTX, as well as MTX, suppressed uptake of tritium-labeled MTX (MTX-3H) by the choroid plexus of a rabbit brain in vitro. Bromine-77-labeled 3'-Br-MTX (3'-77Br-MTX) injected into the femoral artery of a tumor bearing rat leg together with MTX showed high initial retention activity by the tumor. These reports indicate that 3'-Br-MTX can be taken up in organs or cells comparable to MTX. A Yoshida lymphosarcoma line resistant to an alkylating agent showed collateral sensitivity against 3'-Br-MTX. The original Yoshida sarcoma was transplanted into rats, which were then treated with 3'-Br-MTX. Initially, the tumor volume reduced, but tumor regrowth commenced, and less response was seen to the second dose. Some of the 3'-Br-MTX treated tumors were then transplanted into other rats. Treatment of the retransplanted tumors with the alkylating agent reduced tumor successfully. This suggests that 3'-Br-MTX can work as an antitumor reagent as well as or stronger than MTX under certain conditions. 3'-Cl-MTX inhibited both cultured human leukemic lymphoblasts and the corresponding MTX-resistant subline at a similar efficiency to MTX, suggesting that 3'-Cl-MTX works as an antitumor reagent as well as MTX. The present study showed that MTX reacts with HOBr and HOCl to generate 3'-Br-MTX and 3'-Cl-MTX, respectively. Free amino acids suppressed both the HOBr and HOCl reactions, while taurine suppressed the HOCl reaction but not the HOBr reaction. These results suggest that 3'-Br-MTX and 3'-Cl-MTX may be generated from MTX at inflammation sites, although the reactions will be suppressed by coexistent amino acids. If these halogenations occur on MTX administered to humans with inflammation, they may affect the medi-

---

**Table 1.** Effects of Additives on the Reactions of MTX with HOBr

<table>
<thead>
<tr>
<th>Additives</th>
<th>MTX (µM)</th>
<th>3'-Br-MTX (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>2.6 ± 0.7</td>
<td>95.9 ± 1.1</td>
</tr>
<tr>
<td>NH4Cl</td>
<td>50.4 ± 4.9</td>
<td>42.5 ± 4.6</td>
</tr>
<tr>
<td>CH3NH2/HCl</td>
<td>10.3 ± 2.9</td>
<td>87.1 ± 2.6</td>
</tr>
<tr>
<td>(CH3)2NH/HCl</td>
<td>19.1 ± 3.7</td>
<td>77.7 ± 3.8</td>
</tr>
<tr>
<td>(CH3)2N/HCl</td>
<td>0.2 ± 0.3</td>
<td>95.8 ± 0.4</td>
</tr>
<tr>
<td>Gly</td>
<td>0.0 ± 0.0</td>
<td>95.3 ± 1.9</td>
</tr>
<tr>
<td>Lys</td>
<td>59.6 ± 1.0</td>
<td>38.5 ± 0.3</td>
</tr>
<tr>
<td>Met</td>
<td>89.2 ± 0.1</td>
<td>9.0 ± 0.1</td>
</tr>
<tr>
<td>Taurine</td>
<td>95.8 ± 0.1</td>
<td>4.1 ± 0.1</td>
</tr>
<tr>
<td>Urea</td>
<td>98.2 ± 0.1</td>
<td>1.6 ± 0.0</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.9 ± 1.2</td>
<td>95.6 ± 0.7</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>0.7 ± 0.6</td>
<td>94.3 ± 1.1</td>
</tr>
<tr>
<td>CH3COONa</td>
<td>78.4 ± 0.9</td>
<td>4.9 ± 1.0</td>
</tr>
<tr>
<td>CH3NH2</td>
<td>0.7 ± 0.5</td>
<td>93.8 ± 4.8</td>
</tr>
</tbody>
</table>

---

**Table 2.** Effects of Additives on the Reactions of MTX with HOCl

<table>
<thead>
<tr>
<th>Additives</th>
<th>MTX (µM)</th>
<th>3'-Cl-MTX (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>19.1 ± 3.4</td>
<td>64.6 ± 2.5</td>
</tr>
<tr>
<td>NH4Cl</td>
<td>84.0 ± 2.3</td>
<td>3.0 ± 0.2</td>
</tr>
<tr>
<td>CH3NH2/HCl</td>
<td>98.5 ± 0.7</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>(CH3)2NH/HCl</td>
<td>97.3 ± 0.7</td>
<td>2.1 ± 0.2</td>
</tr>
<tr>
<td>(CH3)2N/HCl</td>
<td>29.3 ± 6.0</td>
<td>64.5 ± 3.4</td>
</tr>
<tr>
<td>(CH3)2NCl</td>
<td>23.1 ± 2.2</td>
<td>63.5 ± 2.2</td>
</tr>
<tr>
<td>Gly</td>
<td>99.1 ± 0.5</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>Lys</td>
<td>98.4 ± 0.3</td>
<td>0.7 ± 0.0</td>
</tr>
<tr>
<td>Met</td>
<td>100.0 ± 0.4</td>
<td>0.7 ± 0.0</td>
</tr>
<tr>
<td>Taurine</td>
<td>99.8 ± 0.3</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>Urea</td>
<td>98.8 ± 0.3</td>
<td>0.8 ± 0.0</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>21.5 ± 8.2</td>
<td>62.4 ± 6.6</td>
</tr>
<tr>
<td>Glucose</td>
<td>21.5 ± 8.2</td>
<td>62.4 ± 6.6</td>
</tr>
<tr>
<td>CH3COONa</td>
<td>26.3 ± 9.8</td>
<td>59.7 ± 8.3</td>
</tr>
</tbody>
</table>

---

*a* Concentrations of MTX and 3'-Br-MTX when a solution of 100µM MTX was incubated with 100µM HOBr in 100mM potassium phosphate buffer (pH 7.4) at 37°C for 24h in the presence of 1mM of additives. All the reaction mixtures were analyzed by RP-HPLC. Means ± S.D. (*n* = 3) are presented.
tional efficacy of MTX.

**Experimental**

**Materials**  t-(-)+-Amethopterin (MTX) was purchased from Nacalai Tesque (Kyoto, Japan). NaBr (>99.99%) was purchased from Sigma-Aldrich (MO, U.S.A.). Other chemicals were obtained from Nacalai Tesque or TCI (Tokyo, Japan). Bromide-free hypobromous acid (HOBr) was prepared by the addition of silver nitrate and subsequent distillation, as previously reported. 27,29 The concentration of HOBr was determined spectrophotometrically at 290 nm relative to that of MTX in the mixed solution. The estimated ε_{290} values were 26800 M⁻¹ cm⁻¹ for 3'-Br-MTX and 26500 M⁻¹ cm⁻¹ for 3'-Cl-MTX.

**Conflict of Interest** The authors declare no conflict of interest.

**References**