Inhibition Kinetics of Theophylline Metabolism by Mexiletine and Its Metabolites

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To further characterize the mode of drug interaction between theophylline (TP) and mexiletine (ME), in vitro kinetic studies were carried out using rat liver microsomes and 9000 × g supernatant. The kinetic study revealed that the K_m values and V_max/K_m ratio for the metabolic conversion of TP to 1,3-dimethyluric acid (1,3-DMU) were the second lowest and the highest, respectively, of four metabolic pathways. Thus, the rank of efficiency of the oxidative metabolism by microsomal cytochrome P-450 (P-450) isozymes was TP to 1,3-DMU > TP to 1-methylxanthine (1-MX) > TP to 3-MX > 1,3-DMU to 1-methyluric acid, suggesting that the isozyme metabolizing TP would have a higher affinity for the oxidation at the 8-position in TP molecules than at the 1- and 3-positions. Lineweaver-Burk plots showed that the conversion of TP to 3-MX and to 1,3-DMU was inhibited competitively by ME and its metabolites, and that the pathway of TP to 1-MX was inhibited noncompetitively. In consideration of the K_i values calculated, it seems probable that deamino-α-hydroxy ME (DApHME) might be the most potent inhibitor of the metabolic pathways of TP, and that the rank order of inhibition is approximately DApHME > α-hydroxy ME > deamino-α-hydroxymethyl ME > ME > deamino-α-hydroxymethyl ME, with some exceptions. The mechanism of the interaction between TP and ME is probably due to the metabolic antagonism in the liver, and TP, ME and their metabolites share some of the same metabolic pathways, mediated by P-450 isozymes.

Keywords theophylline-mexiletine interaction; metabolic interaction; oxidative metabolism; metabolic inhibition; inhibition kinetics; rat liver microsome

Theophylline (TP) has been used extensively for many years in the treatment of chronic obstructive pulmonary disease. Because it has a relatively narrow therapeutic index, TP can readily cause a variety of unpleasant and serious side effects. It has been often used with other drugs. TP and mexiletine (ME) occasionally are co-administered to treat patients with chronic or obstructive pulmonary disease and cardiac arrhythmias. An interaction between TP and ME has been reported in patients,1-5 who had a significant increase in serum TP levels after initiation of ME therapy. The mechanism of the interaction between TP and ME is reported to be the inhibition of demethylation6-7 and of α-hydroxylation of TP by ME.7

TP is extensively metabolized by the cytochrome P-450 (P-450) system, and the major metabolites are 1,3-dimethyluric acid (1,3-DMU), 3-methylxanthine (3-MX) and 1-methylxanthine (1-MX).8-11 ME also undergoes extensive hepatic metabolism primarily by oxidation and reductive processes.12,13 The primary metabolites are α-hydroxy ME (pHME) and hydroxymethyl ME (HMME).14,15 Thus, it is supposed that TP and ME may share some of the same metabolic pathways, mediated by P-450.

This study was designed to gain insight into the mechanisms of interaction between TP or its metabolites and ME or its products, and to clarify whether TP and ME share the same metabolic pathways. The in vitro metabolism of TP and its metabolites was measured in the presence of ME and its products using rat liver microsomes, and the inhibition kinetics was analyzed based on the results obtained.

MATERIALS AND METHODS

Chemicals TP was purchased from Tokyo Organic Chemicals (Tokyo, Japan). 1-MX, 3-MX, 1,3-DMU and 1-methyluric acid (1-MU) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). ME [1-(2',6'-dimethylphenoxy-2-amino propane] hydrochloride, pHME, HMME, deamino-hydroxymethyl ME (DAHMME) and deamino-α-hydroxy ME (DApHME) were generous gifts from Nippon-Boehringer Ingerheim Co. (Kawanishi, Japan). NADPH was purchased from Oriental Yeast Co. (Tokyo, Japan). Theobromine, an internal standard for HPLC was obtained from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals used were of reagent grade or HPLC quality.

Preparation of Hepatic 9000 × g Supernatant and Microsomes Animals were fasted for 24 h before sacrifice by decapitation. The hepatic 9000 × g supernatant was prepared by the ordinary method. The microsomes were prepared from the 9000 × g supernatant according to the procedure of Omura and Sato.16

In Vitro Metabolism Experiment The in vitro metabolism of TP and its metabolites was measured as follows. TP or its metabolite at final concentrations of 1.2 to 10 mM, rat liver microsomes (2 mg protein/ml), NADPH (1.2 mM) and 0.1 M phosphate buffer (pH 7.4) were incubated for 30 min in the presence or absence of ME, pHME or DAHMME at final concentrations of 0.5, 1.0, 2.0 and 4.0 mM; of HMME at final concentrations of 0.5, 1.0, 2.0 and 3.0 mM; and of DApHME at final concentrations of 0.1, 0.2, 0.4 and 0.8 mM, in a final volume of 0.5 ml. Reactions were stopped by the addition of 0.5 ml 2% ZnSO_4. After centrifugation, the products in the supernatant were determined by the HPLC method.17

Determination of TP and Its Metabolites TP and its metabolites were determined by the method of Robson et al.,18 with slight modifications. Briefly, 10 μl of the internal standard solution (theobromine, 20 μg/ml) was added to
the reaction mixture. After centrifugation the aqueous mixture was saturated with (NH₄)₂SO₄ and extracted with 5 ml of dichloromethane:isopropanol (80:20, v/v). The extract was evaporated, and the residue redissolved in 0.2 ml mobile phase for chromatographic analysis was injected onto a reversed-phase Chemocorbond 5-ODS-H column (4.6 × 250 mm, Chemco Co., Tokyo, Japan) using a Shimadzu liquid chromatograph (model LC-6A) equipped with an UV spectrophotometer (model SPD-6AV). The mobile phase composition was 2% methanol: 2% acetonitrile: 96% 10 mM sodium acetate (pH 3.5), pumped at a flow rate of 1.0 ml/min. Detection was at 273 nm. The calibration lines were determined by the same method.

**Determination of Protein** Protein concentration was determined by the method described by Lowry et al. with bovine serum albumin as a standard.\[1\]

**Kinetic and Statistical Analyses** The kinetic data were analyzed using a Lineweaver–Burk double reciprocal plot and a Dixon plot. Maximum velocity ($V_{max}$), Michaelis constant ($K_m$), apparent maximum velocity ($V_{p}$), apparent Michaelis constant ($K_p$) and inhibitory constant ($K_i$) were calculated by least squares regression analysis. The $K_m$ and $V_{max}$ calculated in each experiment, were utilized for the determination of $K_i$ value. Best fitting of the data was performed by weighting the reciprocal of their square. The *in vitro* inhibition kinetic analyses of all data were carried out by estimating the three inhibition types, competitive, noncompetitive and mixed type, and the best type was evaluated based on Akaike's information of criterion (AIC), which gave the lowest AIC value.

The means of all data are presented with their standard deviation (mean ± S.D.). Statistical analysis between data points was performed using the one-way analysis of variance (ANOVA) and Dunnnett's test, and $p$ value of 0.05 or less was considered to be significant.

**RESULTS**

**In Vitro Oxidation Studies** Lineweaver–Burk plots describing the kinetics of demethylation and hydroxylation of TP and its metabolites are shown in Fig. 1. The $K_m$ and $V_{max}$ values for the metabolic conversion are summarized in Table I. The $K_m$ value for the conversion of TP to 1-MX was the lowest of the values for the four metabolic pathways, although the $K_m$ for the pathway of TP to 1,3-DMU was the second lowest; conversely, the $V_{max}$ value was the highest in the conversion of TP to 1,3-DMU. The $K_m$ values for the reaction of TP to 3-MX and 1,3-DMU to 1-MU were comparatively high.

The $V_{max}/K_m$ ratios were also estimated as measures of intrinsic enzyme activity. The ratio for the conversion of TP to 1,3-DMU was much larger than those for the formation of 3-MX and 1-MX, suggesting that the former pathway would proceed more rapidly. These results indicated that the P-450 isozyme metabolizing TP would have a higher affinity for the oxidation process at the 8-position than at the 1- and 3-positions, and that the order of the oxidation rate was TP to 1,3-DMU > TP to 1-MX > TP to 3-MX > 1,3-DMU to 1-MU.

**In Vitro Inhibition Kinetic Studies** The inhibition by ME and its metabolites of the oxidation of TP and its metabolites was examined in microsomal fractions. The metabolic conversion of TP to 3-MX was inhibited competitively by ME and its metabolites as shown in Fig. 2. The calculated $K_i$ values are tabulated in Table II. DAPHME gave the lowest $K_i$ value (0.513 ± 0.176 mm), suggesting it is a potent inhibitor of the pathway, whereas HMME yielded the highest (4.38 ± 2.78 mm). The rank order of the $K_i$ value follows, in a descending manner: DAPHME > DAPHMME > pHME > ME > HMME.

The metabolic pathway from TP to 1-MX was inhibited noncompetitively by ME and its metabolites (Fig. 3), which yielded comparatively low $K_i$ values compared with those obtained in the conversion of TP to 3-MX (Table III). DAPHME also gave the lowest $K_i$ estimate and ME had the highest of them.

ME and its metabolites inhibited competitively the hydroxylation of TP to 1,3-DMU (Fig. 4). The calculated $K_i$ values also suggested that DAPHME would be the most potent inhibitor and that pHME was a comparatively potent one of the pathway (Table IV). The rank order of inhibition was approximately DAPHME > pHME > ME > DAPHMME > HMME. The inhibitory tendency was roughly similar to that obtained in the inhibitory study of TP to 3-MX, with some exceptions.

The validity of $K_i$ values obtained by Lineweaver–Burk plots were evaluated by means of Dixon plots, and the values were similar to those obtained by Lineweaver–Burk plots, *e.g.* in the conversion of TP to 3-MX, the $K_i$...
values were 4.028 ± 2.101 (by ME), 1.490 ± 0.538 (by pHME), 5.264 ± 2.068 (by HMME) and 0.349 ± 0.104 (by DApHME).

Formal kinetic studies for the inhibition of the pathway from 1,3-DMU to 1-MU were carried out. Although the reaction was inhibited by ME and its metabolites to an appreciable extent, considerable variances were noted in calculated $K_i$ values (data not shown). Therefore, the results are shown as the remaining activity after treatment with ME and its metabolites in Table V. DApHME also showed relatively potent inhibition of the 3-oxidation of 1,3-DMU to 1-MU.

The conversion of 1-MX to 1-MU, which is mediated by the soluble enzyme, xanthine oxidase, was tested in the presence of ME or its metabolites using the 9000 × g supernatant of liver. No inhibition of the metabolic pathway was seen in this study, and conversely only slight enhancement was observed by HMME.

DISCUSSION

The interaction between ME and TP is most likely caused by an inhibition in the activity of P-450 isozymes that mediates the metabolism of TP. However, the contribution of ME metabolites to the metabolic pathways of TP is not yet known. To further characterize the mode of the drug interaction, in vitro kinetic studies were carried out in rat liver microsomes and the 9000 × g supernatant.

Because the $K_m$ values are representative of the affinity of a substrate toward an enzyme and $V_{max}/K_m$ ratios are used as measures of intrinsic enzyme activity, these calculated values were compared. The $K_m$ value for the metabolic conversion of TP to 1,3-DMU (8-hydroxylation) was low. In addition, the $V_{max}/K_m$ ratio was much higher than those for the conversion of TP to 3-MX, TP to 1-MX and 1,3-DMU to 1-MU. These results demonstrate that the formation of 1,3-DMU from TP...
proceeded more easily than the three other oxidation processes, TP to 1-MX, TP to 3-MX and 1,3-DMU to 1-MU. The most likely explanation for the present findings are either that the pathway of TP to 1,3-DMU is related to one P-450 isozyme, different from the isozyme involved in the metabolism from TP to 3-MX and to 1-MX and from 1,3-DMU to 1-MU, or that the isozyme would have a higher affinity for the oxidation process at the 8-position in TP molecules than that at the 1- and 3-positions. It is suggested that TP is metabolized by at least two isozymes of P-450: one predominantly performing the N-demethylation and the other the 8-hydroxylation.\(22^{24}\) McManus et al. stated that the 8-hydroxylation of TP to 1,3-DMU was the major pathway and that in rat liver microsomes this metabolite accounted for 94% of the total metabolites.\(25\) It is now clear from studies with human P-450 isozymes that P-450 CYP1A2 is able to convert TP to 1,3-DMU, 1-MX and 3-MX.\(26\) Sarkar et al. concluded that both N-demethylations (TP to 3-MX and TP to 1-MX) of TP in human liver microsomes are predominantly conducted by human forms immunochemically related to rat P-450c and P-450d.\(27\) Additionally, they called into question the assumption that 3-MX or 1-MX formation is conducted by a common P-450 isozyme. Judging from our results and the data reported, it would be reasonable to assume that the main metabolic pathways of TP are the conversion of TP to 1,3-DMU (hydroxylation at position 8) and TP to 1-MX (demethylation at position 3) in rat, although 1-MX is rapidly metabolized by xanthine oxidase\(19,20\) (Fig. 5); it is also reasonable to assume that an isozyme would have a high affinity for the oxidation at the 8-position in TP. This is also supported by our previous findings that high plasma concentrations of 1,3-DMU and 1-MU and the comparatively rapid elimination of both metabolites from the circulation were observed after intravenous administration of aminophylline to rat, with

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**Table III. Inhibitory Constants of 3-Demethylation of TP to 1-MX**

<table>
<thead>
<tr>
<th>ME</th>
<th>pHME</th>
<th>HMME</th>
<th>DAPHME</th>
<th>DAHMME</th>
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<tbody>
<tr>
<td>(V_{m}^{a}) (pmol/min/mg protein)</td>
<td>14.0 ± 7.0</td>
<td>9.4 ± 3.2</td>
<td>14.8 ± 0.5</td>
<td>15.5 ± 3.7</td>
</tr>
<tr>
<td>(V_{m}^{b}) (pmol/min/mg protein)</td>
<td>11.0 ± 4.0</td>
<td>6.1 ± 3.6</td>
<td>11.6 ± 1.4</td>
<td>11.7 ± 2.3</td>
</tr>
<tr>
<td>(K_{m}) (mm)</td>
<td>0.593 ± 0.201</td>
<td>0.270 ± 0.086</td>
<td>0.559 ± 0.152</td>
<td>0.086 ± 0.022</td>
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</table>

\(a\) TP + 0.5 mm ME, pHME, DAHMME, HMME or 0.1 mm DAPHME.  
\(b\) TP + 1.0 mm ME, pHME, DAHMME, HMME or 0.2 mm DAPHME.  
\(c\) TP + 2.0 mm ME, pHME, DAHMME, HMME or 0.4 mm DAPHME.  
\(d\) \(p<0.01\) compared with ME, HMME and DAHMME.  
\(e\) \(p<0.05\) compared with pHME. Each value represents the mean ± S.D. \((n=3-5)\).
Fig. 4. Lineweaver-Burk Plots Showing Inhibition of Microsomal 1,3-DMU Formation from TP by ME (A), pHME (B), HMME (C), DApHME (D), and DAHMME (E).

Inhibitor concentration was zero (○), 0.5 mm (▲), 1.0 mm (■) and 2.0 mm (●), except for DApHME [0.1 mm (△), 0.2 mm (□) and 0.4 mm (●)]. Each point represents the mean ± S.D. (n = 3–5).

### Table IV. Inhibitory Constants of 8-Oxidation of TP to 1,3-DMU

<table>
<thead>
<tr>
<th></th>
<th>ME</th>
<th>pHME</th>
<th>HMME</th>
<th>DApHME</th>
<th>DAHMME</th>
</tr>
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<tbody>
<tr>
<td>K&lt;sub&gt;1&lt;/sub&gt; ( \text{(mm)} )</td>
<td>5.9 ± 1.1</td>
<td>4.8 ± 1.7</td>
<td>5.1 ± 0.7</td>
<td>4.3 ± 1.4</td>
<td>5.8 ± 0.5</td>
</tr>
<tr>
<td>K&lt;sub&gt;2&lt;/sub&gt; ( \text{(mm)} )</td>
<td>7.3 ± 1.2</td>
<td>10.4 ± 3.7</td>
<td>6.2 ± 0.8</td>
<td>5.1 ± 1.8</td>
<td>7.9 ± 1.3</td>
</tr>
<tr>
<td>K&lt;sub&gt;3&lt;/sub&gt; ( \text{(mm)} )</td>
<td>8.8 ± 3.1</td>
<td>15.7 ± 7.3</td>
<td>7.5 ± 1.2</td>
<td>7.0 ± 3.2</td>
<td>10.1 ± 2.2</td>
</tr>
<tr>
<td>K&lt;sub&gt;4&lt;/sub&gt; ( \text{(mg/ml)} )</td>
<td>0.526 ± 0.261</td>
<td>0.425 ± 0.177</td>
<td>0.970 ± 0.498&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.186 ± 0.165&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.728 ± 0.368&lt;sup&gt;a&lt;/sup&gt;</td>
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<sup>a</sup> TP + 0.5 mm ME, pHME, DAHMME, HMME or 0.1 mm DApHME.  
<sup>b</sup> TP + 1.0 mm ME, pHME, DAHMME, HMME or 0.2 mm DApHME.  
<sup>c</sup> TP + 2.0 mm ME, pHME, DAHMME, HMME or 0.4 mm DApHME.  
<sup>d</sup> P < 0.05 compared with ME and pHME.  
<sup>e</sup> P < 0.05 compared with ME, HMME and DAHMME.

Each value represents the mean ± S.D. (n = 3–5).

### Table V. Inhibition of 3-Oxidation of 1,3-DMU to 1-MU in Rat Liver Microsomes

<table>
<thead>
<tr>
<th>Remaining activity (%)</th>
<th>ME</th>
<th>pHME</th>
<th>HMME</th>
<th>DApHME</th>
<th>DAHMME</th>
</tr>
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<tbody>
<tr>
<td>1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>66.4 ± 16.7</td>
<td>62.5 ± 6.2</td>
<td>88.7 ± 12.6</td>
<td>83.9 ± 16.4</td>
<td>74.0 ± 26.6</td>
</tr>
<tr>
<td>2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>54.5 ± 11.5</td>
<td>38.6 ± 9.2</td>
<td>62.4 ± 40.1</td>
<td>53.3 ± 19.7</td>
<td>50.7 ± 18.0</td>
</tr>
<tr>
<td>3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>71.1 ± 42.9</td>
<td>54.2 ± 27.2</td>
<td>75.5 ± 18.6</td>
<td>15.2 ± 9.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>69.2 ± 42.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> 1,3-DMU + 0.5 mm ME, pHME, DAHMME, HMME or 0.1 mm DApHME. 
<sup>b</sup> 1,3-DMU + 2.0 mm ME, pHME, DAHMME, (1.0 mm) HMME or 0.4 mm DApHME. 
<sup>c</sup> The activity with inhibitor/activity without inhibitor (%) in mm concentration of inhibitor. 
<sup>d</sup> P < 0.05 compared with ME, HMME and DAHMME. Each value represents the mean ± S.D. (n = 3–10).

A negligible detection of 3-MX and 1-MX.  

The in vitro inhibitory kinetic studies demonstrated that ME and its metabolites inhibited competitively the pathway of TP to 3-MX and to 1,3-DMU, and non-competitively the metabolism of TP to 1-MX (Figs. 2, 3 and 4). Additionally, the calculated K<sub>i</sub> values indicated that DApHME and pHME extensively inhibited the three metabolic pathways of TP, and that HMME which is a metabolite oxidized at the 2'-position was a relatively less potent inhibitor of the pathways. These results suggest that the oxidation process of methyl substituent at the 2'-position in ME molecule would probably be involved in the marked inhibition of TP metabolism by P-450, and, moreover, that the aromatic hydroxylation process at the para-position in the molecule was relatively strongly related to the inhibition. Thus, ME metabolites, particularly DApHME and pHME, may successfully compete for existing metabolic pathways of TP, by binding to the active site of the isozyme(s) that oxidizes TP. Substrates oxidized by the same form of P-450 should act as competitive inhibitors of each other's metabolism. The major pathway of ME biotransformation in humans is hydroxylation followed by secondary methylation, reduc-
tion and conjugation (Fig. 6).\textsuperscript{12,13} Thus, it is possible that ME and TP or their metabolites share some of the same metabolic pathways.

The difference in $K_i$ values between ME and its metabolites is probably due to the difference in their affinity towards P-450 isozymes based on the position and polarity of substituents. The fact that the $K_i$ values of DApHME and pHME were generally smaller than those of HMME and DAHMME suggests that the $p$-hydroxy group on phenyl residue in the metabolites may weaken the binding of the corresponding substrate to the enzyme, probably due to the steric hindrance.

The noncompetitive inhibition observed in the metabolic conversion of TP to 1-MX may be due to the involvement of two kinetically different isozymes such as P-450c and P-450d in this oxidation process, in which each isozyme undergoes a different inhibition by ME and its metabolites. Consequently, the inhibition type might be non-competitive.

In man, HMME and pHME are considered as major metabolites of ME,\textsuperscript{24,25} and both metabolites and their corresponding alcohols (DAHMME and DApHME) are excreted in urine in conjugated and free forms, accounting for up to 20% of the dose.\textsuperscript{12} These data suggest that ME and its major metabolites DApHME and pHME, which are potent inhibitors, may strongly inhibit the in vivo
metabolism of TP and thereby induce a decrease in plasma clearance of TP and its metabolites. This effect indicates that concurrent administration of the two drugs may require reduction in the dose of TP to minimize the risk of toxicity.

The therapeutic concentration of TP and ME in plasma is 55–110 mM and 2.8–8.4 μM, respectively. The concentrations used in this study were much higher than the therapeutic concentrations, however, the inhibition of TP metabolism by ME has already been demonstrated by many studies. Our study should clarify the inhibitory mechanism operating between TP and ME or its metabolites, probably in the therapeutic dose.

In conclusion, the present kinetic studies revealed that the rank of efficiency of the oxidative metabolism of TP by microsomal P-450 isozymes was TP to 1,3-DMU > TP to 1-MX > TP to 3-MX > 1,3-DMU to 1-MU. Lineweaver–Burk plots showed that the conversion of TP to 3-MX and to 1,3-DMU was inhibited competitively by ME and its metabolites, and that the pathway of TP to 1-MX was inhibited noncompetitively. Considering the Kᵢ values, it is probable that DApHME was the most potent inhibitor of the metabolic pathways of TP, and the rank order of inhibition was approximately DApHME > pHME > DAHMME > ME > HMME, with some exceptions.

REFERENCES