Metabolism of theophylline by cDNA-expressed human cytochromes P-450

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1 Theophylline metabolism was studied using seven human cytochrome P-450 isoforms (CYPs), namely CYP1A1, 1A2, 2A6, 2B6, 2D6, 2E1 and 3A4, and microsomal epoxide hydroxylase (EH), expressed in human B-lymphoblastoid cell lines.

2 At a high theophylline concentration of 10 mM four CYPs (1A1, 1A2, 2D6, 2E1) catalyzed the metabolism of theophylline.

3 Theophylline had the highest affinity (apparent Km range 0.2–1.0 mM) for the CYP1A subfamily and the kinetics of metabolic formation mediated by CYP1A2 indicated substrate-inhibition (Ki range 9–16 mM).

4 CYP1A2 catalyzed the demethylation of theophylline as well as its hydroxylation, and was associated with the highest intrinsic clearance (1995 l h⁻¹ per mol CYP) to 1,3-dimethyluric acid (DMU). Therefore, this isoform can be considered to be the most important enzyme involved in theophylline metabolism in vitro.

5 CYP2E1 was responsible for a relatively high intrinsic clearance by 8-hydroxylation (289 l h⁻¹ per mol CYP). The apparent Km value of this reaction was about 15 mM, suggesting that CYP2E1 may be the low-affinity high-capacity isoform involved in theophylline metabolism.

6 The affinity of theophylline for CYP1A1 was comparable with that of its homologue 1A2. When induced, the participation of CYP1A1 in theophylline metabolism may be important.

7 CYP2D6 played only a minor role and CYP3A4 was not active in the in vitro metabolism of theophylline.

8 Our findings confirm the major role of CYP1A2 in theophylline metabolism and explain why in vivo the elimination kinetics of theophylline are non-linear and in vitro theophylline metabolism by human liver microsomes does not obey monophasic kinetics.

9 The data suggest also that not only tobacco smoking but also chronic alcohol intake may influence theophylline elimination in man as ethanol induces CYP2E1.

Keywords theophylline metabolism CYP1A2 CYP2E1 in vitro cDNA expressed microsomes

Introduction

It is well recognized that the metabolism of theophylline is influenced by many variables including genetic factors [1], smoking [2–4], disease [5, 6] and drugs [7]. In man theophylline is biotransformed principally in the liver by the microsomal mixed-function oxide system (MFO) to 1,3-dimethyluric acid (DMU), 3-methylxanthine (3MX) and 1-methyluric acid (1MU) (Figure 1), and these metabolites are eliminated predominantly by the kidneys. In human urine unchanged drug, DMU, 1MU and 3MX represent 13, 48, 20 and 13%, respectively of the eliminated amount [8–10]. Among the primary

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metabolites, DMU is clearly the main product. 1-methylxanthine (1MX), which is formed primarily by 3-demethylation, is present in a high concentration in in vitro studies [11–13] but only represents 4.6% of the urinary recovery because in vivo it is rapidly converted to 1MU by xanthine oxidase [8]. Indirect evidence suggests that theophylline is metabolized by more than one CYP, including CYP1A2 [11–14]. Using a high substrate concentration (20 mM) Sarkar et al. [14] observed that CYPs 1A2 and 2E1, but not CYP3A3, contributed to theophylline metabolism in human microsomes. However, at such a high substrate concentration the authors were not able to distinguish the activities of high- and low-affinity CYPs involved in the reaction. Gu et al. [15] incubated theophylline (1 mM) with cDNA expressed human CYPs and found that CYPs 1A2, 2E1 and 3A4 contributed to its metabolism. Fuhr et al. [16] found exclusive metabolism of theophylline to DMU using cell lines expressing human CYP1A2. Thus, the qualitative and quantitative aspects of the involvement of various CYPs in the metabolism of theophylline are unclear.

The aims of the present study were to identify the CYP(s) involved in the metabolism of theophylline using microsomes prepared from human B-lymphoblastoid cell lines stably transfected with different human CYPs and epoxide hydroxylase, and to clarify their relative contributions to theophylline metabolism in vivo.

**Methods**

**Chemicals and biological materials**

Theophylline (1MX), 1-methylxanthine (1MX), 3-methylxanthine (3MX), 1,3-dimethyluric acid (DMU) and the internal standard theobromine were obtained from Sigma Chemical Co. (St Louis, MO 63178, USA). 8-[14C]-theophylline, with a specific activity of 58 mCi mmol⁻¹, was purchased from Amersham (Rahn Co. Zürich, Switzerland). All other chemicals and solvents were of analytical grade. Microsomal preparations from transfected human B-lymphoblastoid cell lines coding for human CYP1A1, 1A2, 2A6, 2B6, 2D6-Met, 2E1, 3A4 and epoxide hydroxylase were purchased from Gentest Co. (Woburn, MA 01801 USA). The microsomal preparations were received frozen and were stored at −75°C until use. The protein concentration was 10 mg ml⁻¹. The specifications of each CYP system are summarized in Table 1. CYP contents were measured by the method of Omura & Sato [17].

**Purification of theophylline by liquid chromatography**

Commercially available theophylline was purified by h.p.l.c. as described by Robson et al. [12].

**H.p.l.c. assay of theophylline and its metabolites**

Theophylline metabolites were assayed by a modification of the h.p.l.c. method of Campbell et al. [11]. A reversed phase h.p.l.c. column (Lichro CART 125 × 4.6 mm, MERK Co., CH-8953 Dietikon, Switzerland) maintained at 37°C was used with a mobile phase of tetrahydrofuran:acetonitrile:acetic acid 5 mM (0.1:0.2:99.7 v:v) at a flow rate of 1.5 ml min⁻¹. The metabolites of theophylline were detected at 280 nm using a u.v. detector. The internal standard

![Figure 1](image)

**Figure 1** Main metabolic pathways of theophylline.

Table 1 Expression levels and catalytic activities of cDNA expressed human CYP isoforms (human B-lymphoblastoid cell lines)

<table>
<thead>
<tr>
<th>CYP</th>
<th>Lot number</th>
<th>CYP content (pmol mg⁻¹)</th>
<th>Substrate</th>
<th>Activity (pmol min⁻¹ mg⁻¹ protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A1</td>
<td>9</td>
<td>25</td>
<td>EROD</td>
<td>120</td>
</tr>
<tr>
<td>1A2</td>
<td>17</td>
<td>40</td>
<td>EROD</td>
<td>69</td>
</tr>
<tr>
<td>2A6</td>
<td>14</td>
<td>55</td>
<td>Coumarin</td>
<td>769</td>
</tr>
<tr>
<td>2B6</td>
<td>10</td>
<td>60</td>
<td>EFC</td>
<td>150</td>
</tr>
<tr>
<td>2D6-Met*</td>
<td>29</td>
<td>160</td>
<td>Bufuralol</td>
<td>870</td>
</tr>
<tr>
<td>2E1*</td>
<td>13</td>
<td>40</td>
<td>p-nitrophenol</td>
<td>760</td>
</tr>
<tr>
<td>3A4*</td>
<td>1</td>
<td>20</td>
<td>Testosterone</td>
<td>1100</td>
</tr>
<tr>
<td>Epoxide hydrolase</td>
<td>4</td>
<td>—</td>
<td>BZP</td>
<td>150</td>
</tr>
</tbody>
</table>

BZP: benzo(a)pyrene, EFC: 7-ethoxy-4-trifluoromethylcoumarin, EROD: 7-ethoxyresorufin.

*With reductase.
(theobromine) was added to incubation mixtures just before extraction.

**Microsomal incubations**

Theophylline (0.04–25 mM) in 0.1 M sodium phosphate buffer, pH 7.4 and an NADPH generating system (0.5 IU isocitrate dehydrogenase, 3 mM NADP, 5 mM sodium isocitrate and 5 mM MgCl₂) were made up to a volume of 0.5 ml in 1.5 ml polypropylene tubes. After preincubation for 2 min at 37°C, the reaction was started by addition of 0.25 mg microsomal protein previously maintained at 4°C. After incubating at 37°C for 3 h under air, the reaction was stopped by addition of 0.050 ml 2M HCl and 7 ml dichloromethane:isopropyl alcohol (80:20 v/v). Internal standard (500 ng theobromine dissolved in water) and 0.5 g ammonium sulphate were added to the reaction mixture. The tube was capped and shaken for 10 min (Labshaker, Basel, Switzerland). After centrifugation at 1000 g for 5 min, the organic phase was transferred to a conical test tube and evaporated to dryness under nitrogen at 37°C. The residue was dissolved in 150 μl of mobile phase and 120 μl were injected onto the h.p.l.c. In experiments using recombinant CYP2A6, the phosphate buffer was replaced by Tris buffer 0.1 M pH 7.4, because phosphate buffer inhibits the activity of this CYP.

Enzyme kinetics were determined in duplicate incubations performed on the same day using nine theophylline concentrations (0.03 to 10 mM) for CYP1A1, nine concentrations (0.03 to 9 mM) for CYP1A2, nine concentrations (0.7 to 25 mM) for CYP2D6 and 16 concentrations (0.04 to 25 mM) for CYP2E1. For substrate concentrations below 0.15 mM [8-14C]-theophylline was used. When radio labelled theophylline was used the chromatographic fractions corresponding to each theophylline metabolite peak were collected and counted in a liquid scintillation counter (Packard Co., TR1900, Downers Grove, IL 60515, USA).

**Data analysis**

Reaction rates were fitted by non-linear regression assuming Michaelis-Menten kinetics or substrate-inhibition enzyme kinetics [18] using the ProFit software package (QuantumSoft, 8023 Zürich, Switzerland).

Based on the enzyme kinetic parameters the amount of each theophylline metabolite produced per hour and mol CYP by individual CYPs over the concentration range of 0.01–100 mM was calculated. The values were then normalized for the relative abundance of each CYP in human liver as determined by Shimada et al. [19]. Thus, the average specific contents of CYPs 1A2, 2E1, 2D6 were assumed to be 13, 7 and 2%, respectively, of total cytochrome P450. Because the specific content of inducible CYP1A1 is not known, it was set arbitrarily at 1%. Thus, the contribution of each individual CYP to the formation of a given metabolite can be estimated and expressed as a percentage of total formation.

**Results**

The limit of determination of all of the measured theophylline metabolites was 2 μM, and between- and within-day coefficients of variation of the assays were less than 5% in the concentration range 50–1000 ng ml⁻¹ and 8–14% for concentrations below 50 ng ml⁻¹. The initial rate of theophylline metabolism was linear with microsomal protein concentration (10–100 μg) over 4 h. Therefore, 50 μg microsomal protein per assay and an incubation time of 3 h were used in all experiments.

At a substrate concentration of 10 mM, CYPs 1A1, 1A2, 2D6, and 2E1 catalyzed theophylline biotransformation to demethylated and 8-hydroxylated metabolites, whereas the production of metabolites in incubations containing CYPs 2A6, 2B6, microsomal epoxide hydrolase or control cell microsomes containing only vector was below the assay detection limit. The CYP3A4 expression system was shown to metabolize caffeine and nimodipine, but did not show any significant activity with respect to theophylline metabolism.

The formation of theophylline metabolites by CYPs 1A1, 2D6, 2E1 obeyed Michaelis-Menten enzyme kinetics, whereas metabolite formation mediated by CYP1A2 was better described by a substrate-inhibition model [18] (Figure 2). Estimates of the kinetic parameters and their standard deviation (s.d.) for each CYP are shown in Table 2. The data show that theophylline has a particularly high affinity for the CYP1A subfamily. The apparent $K_m$ and $K_i$ values were in the range of 0.2–1 and 7–14 mM, respectively. The apparent $K_m$ value for 3MX formation by CYP1A2 was somewhat greater than those for 1MX and DMU but was in the same range when the standard deviation of the estimates was considered. CYP1A1, which may be expressed extrahepatically [20] as well as in some human livers [21], was also shown to catalyze the metabolism of theophylline, but only to 1MX. Furthermore, CYP2E1 was shown...
Table 2  Metabolism of theophylline by cDNA expressed human CYP isoforms.

<table>
<thead>
<tr>
<th>Kinetic parameters</th>
<th>IA1</th>
<th>Cytochrome P-450 isoforms</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Methylxanthine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_m$ $^{a}$</td>
<td>0.31 (0.05)*</td>
<td>0.38 (0.18)* 6.96 (2.16)*</td>
</tr>
<tr>
<td>$V_{max}$ $^{b}$</td>
<td>13.1 (0.8)*</td>
<td>360 (40)* 108 (22)*</td>
</tr>
<tr>
<td>$K_i$ $^{c}$</td>
<td>—</td>
<td>9.6 (0.5)* —</td>
</tr>
<tr>
<td>$CL_{int}$ $^{d}$</td>
<td>42</td>
<td>947 16</td>
</tr>
<tr>
<td>3-Methylxanthine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_m$ $^{a}$</td>
<td>—</td>
<td>1.09 (0.61)* — —</td>
</tr>
<tr>
<td>$V_{max}$ $^{b}$</td>
<td>148 (38)*</td>
<td>—</td>
</tr>
<tr>
<td>$K_i$ $^{c}$</td>
<td>7.1 (0.9)*</td>
<td>—</td>
</tr>
<tr>
<td>$CL_{int}$ $^{d}$</td>
<td>136</td>
<td></td>
</tr>
<tr>
<td>Dimethyluric acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMU</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_m$ $^{a}$</td>
<td>—</td>
<td>0.23 (0.08)* 8.07 (1.20)* 15.3 (1.7)*</td>
</tr>
<tr>
<td>$V_{max}$ $^{b}$</td>
<td>439 (8)*</td>
<td>478 (42)* 4120 (463)*</td>
</tr>
<tr>
<td>$K_i$ $^{c}$</td>
<td>14.1 (1.5)*</td>
<td>—</td>
</tr>
<tr>
<td>$CL_{int}$ $^{d}$</td>
<td>1995</td>
<td>60 269</td>
</tr>
</tbody>
</table>

*Mean ± s.d.
$^{a}$ Apparent $K_m$ (mM).
$^{b}$ Apparent $V_{max}$ (mol h$^{-1}$ mol$^{-1}$ CYP).
$^{c}$ Apparent $K_i$ (mM).
$^{d}$ Intrinsic clearance = $V_{max}/K_m$ (ml h$^{-1}$ mol$^{-1}$ CYP).

*Below detection limit.

to contribute to theophylline metabolism by hydroxylation at C8 leading to the same main metabolite DMU as produced by CYP1A2. This reaction had relatively high $V_{max}$ (4120 mol h$^{-1}$ mol$^{-1}$ CYP) and $K_m$ (15.3 mM) values. It was also observed that CYP2D6 catalyzed the conversion of theophylline to 1MX and DMU. The CYP2D6 cDNA used was that reported by Gonzalez et al. [22] (Gentest Co.’s code: CYP2D6-Met). The apparent $K_m$ values of the CYP2D6-mediated reactions were in the range of 7–8 mM and intrinsic formation clearance of the main metabolite DMU (60 ml h$^{-1}$ mol$^{-1}$ CYP) was only about 3 and 22%, respectively, of that mediated by CYP1A2 and 2E1.

Using the data on relative abundance of human liver CYPs it was predicted that at theophylline concentrations below 0.1 mM (18 μg ml$^{-1}$), CYP1A2 is the most active isoform catalyzing theophylline by 8-hydroxylation, although, at higher substrate concentrations CYP2E1 may contribute (Figure 3a). In contrast to 8-hydroxylation, 3-demethylation is predicted to be mediated almost exclusively by CYP1A over the substrate concentration range 0.01–40 mM (Figure 3b). Thus, the data suggest that theophylline 1-demethylation is catalyzed in vivo specifically by CYP1A2 and that CYP1A1 (when not induced) and CYP2D6 contribute to only a small extent.

Discussion

Among the eight CYPs tested, only CYP1A2 was found to catalyze all of the demethylations and hydroxylation of theophylline. This isoform was associated with the highest intrinsic clearance, in
particular for the formation of the major metabolite DMU and may, therefore, be considered as the main CYP involved in theophylline metabolism. As proposed by Sarkar et al. [14], CYP1A1 may also metabolize theophylline in human liver microsomes, but the participation of this isozyme in their studies was difficult to evaluate because the amount of CYP1A1 in human liver is small. In the present study, CYP1A1 only weakly catalyzed the N3-demethylation of theophylline to IMX. It has been shown that the content of CYP1A1 varies widely in human liver [21] and it may be expressed extrahepatically, especially in the lung [20]. However, it is possible that isozymes other than CYP1A1 are involved in theophylline metabolism in the lung, because human lung microsomes convert theophylline exclusively to DMU [23]. An increase in the urinary recovery of theophylline metabolites in the urine of smokers is consistent with the inducibility of CYP1A1 isozymes by tobacco smoke [24]. Our data reveal also that the substrate dependent formation kinetics of theophylline metabolites mediated by CYP1A2 in vitro may be better described by substrate-inhibition enzyme kinetics than by classical Michaelis-Menten kinetics. In man theophylline elimination kinetics are also non-linear [11, 12]. Therefore, it is possible that the substrate-inhibition phenomenon may also occur in vivo.

Previous investigations have shown that cigarette smoking induces CYP1A2 [25] and influences theophylline demethylation to a greater extent than hydroxylation [2–4]. Therefore, it is possible that more than one CYP isozyme contributes to theophylline metabolism in man. Sarkar et al. [14] and Gu et al. [15] showed that CYPs 1A2 and 2E1 may metabolize theophylline in vitro. With its relatively high apparent $K_m$ value, CYP2E1 may act as the low-affinity enzyme observed previously in in vitro studies [8, 13–15].

Our data may clarify some aspects of drug interactions with theophylline. Cimetidine [26], mexiletine [27], propranolol [28] and ciprofloxacin [29] inhibit the demethylation of theophylline to a greater extent than its hydroxylation, and the converse is true for disulfiram [30]. The differential effects on theophylline pathways may be explained. If CYP2E1 contributes to hydroxylation of theophylline as a low-affinity high-capacity enzyme in vivo the 30% decrease in theophylline clearance observed by Loi et al. [30] in the presence of disulfiram is understandable. This hypothesis is supported by our in vitro data, since the contribution of CYP2E1 to DMU formation is predicted to begin to increase in the therapeutic range of plasma concentrations (5 and 20 $\mu$g ml$^{-1}$ or 0.03–0.11 mM) [31]. Furthermore, since CYP2E1 is induced by chronic ethanol intake [32–34], our data would predict the increase in theophylline clearance observed in alcoholics by Jusko et al. [35]. This is of particular relevance, as up to 50% of alcoholics have evidence of chronic obstructive pulmonary disease [36] and require theophylline therapy.

The comparison of apparent intrinsic clearance values suggests that the contribution of CYP2D6 to theophylline metabolism in vivo is of little importance.

With regard to the role of CYP3A4 in theophylline metabolism, Gu et al. [15] have reported that theophylline hydroxylation is also mediated by this isozyme and its activity was about 20% of that of CYP1A2 and 2E1. However, in the present study, although the CYP3A4 expression system was capable of metabolizing caffeine and nimodipine, it did not show any significant activity with respect to theophylline. This observation is in agreement with Sarkar's data [14].

In summary, our studies confirm that at least four CYPs participate in human theophylline metabolism. They confirm also the dominant role of CYP1A2, with CYP2E1 acting as a low-affinity, high-capacity isozyme contributing to the production of the main theophylline metabolite DMU.

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The terms ethanol and alcohol are used interchangeably in this paper.

References

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