

Clomipramine *N*-demethylation metabolism in human liver microsomes¹

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KEY WORDS clomipramine; cytochrome P-450; furafylline; troleandomycin; mephenytoin; ditiocarb; sulfaphenazole; quinidine; liver microsomes

AIM: To study the effect of cytochrome P-450 (CYP450) inhibitors on clomipramine (Clo) *N*-demethylation *in vitro*. **METHODS:** The kinetic parameters of Clo *N*-demethylation in human liver microsomes were obtained by the Michaelis-Menten equation. The parameters after pretreatment with putative inhibitors of various CYP450 isoforms were compared with controls. **RESULTS:** K_{m1} , K_{m2} , V_{max1} , V_{max2} , V_{max1}/K_{m1} , and V_{max2}/K_{m2} were (0.11 ± 0.06) , $(24 \pm 14) \mu\text{mol} \cdot \text{L}^{-1}$, (114 ± 47) , $(428 \pm 188) \text{nmol} \cdot \text{g}^{-1} \cdot \text{min}^{-1}$, (1.8 ± 1.6) , and $(0.019 \pm 0.005) \text{L} \cdot \text{g}^{-1} \cdot \text{min}^{-1}$, respectively. The interindividual variations for the last 4 parameters reached up to 2.5-, 7.3-, 3.4-, and 1.8-fold. At $5 \mu\text{mol} \cdot \text{L}^{-1}$ of Clo, troleandomycin (Tro), furafylline (Fur), ditiocarb sodium (Dit), and *S*-mephenytoin (Mep) produced a marked inhibition on Clo *N*-demethylation while sulfaphenazole (Sul) and quinidine (Qui) had only slight effects. The inhibitory rates by Dit 30, Mep 500, Fur 10, Tro 10, Fur 80, Tro 200 and Fur 80 + Tro 200 $\mu\text{mol} \cdot \text{L}^{-1}$ were 27.0%, 32.9%, 42.8%, 40.5%, 63.9%, 66.4%, and 78.3%, respectively. The IC_{50} (95% confidence limits) for Fur and Tro were 27.7 (19.1 - 36.3) and 42.1 (20.9 - 63.3) $\mu\text{mol} \cdot \text{L}^{-1}$, respectively. **CONCLUSIONS:** The *N*-demethylation of Clo exhibited a biphasic behavior. This reaction was mediated mainly by both CYP1A2 and CYP3A4, to a minor extent by CYP2C19 at the low concentration of Clo *in vitro*.

Clomipramine (Clo), a tricyclic anti-depressant, undergoes mainly *N*-demethylation and aromatic 8-hydroxylation. *N*-Demethylation is the most important metabolic route leading to the active metabolite desmethylclomipramine (Des), which is further 8-hydroxylated and *N*-demethylated. The 8-hydroxylations of Clo and Des are catalyzed almost entirely by CYP2D6^[1,2], whereas the *N*-demethylation of Clo is catalyzed by other cytochrome isozymes which possibly are CYP2C19^[2-6], CYP1A2^[4,5], and CYP3A4^[5].

Furafylline (Fur), troleandomycin (Tro), ditiocarb sodium (Dit), sulfaphenazole (Sul), and Quinidine (Qui) are selective inhibitors of CYP1A2, 3A4, 2E1, 2C9/10, and 2D6, respectively^[7]. Their concentration ranges at which the activity of CYP1A2, 3A4, 2E1, 2C9/10, and 2D6 is inhibited by more than 70% are 20 - 40, 50 - 200, 15 - 30, 1.0 - 5.0, and 1.0 - 10 $\mu\text{mol} \cdot \text{L}^{-1}$, respectively^[8]. Mephenytoin (Mep) is a probe drug and competitive inhibitor of CYP2C19. The present study was to assess the relative contribution of CYP1A2, 3A4, 2C19, 2C9/10, 2E1, and 2D6 to Clo *N*-demethylation in human liver microsomes at the near-therapeutic concentration of Clo.

MATERIALS AND METHODS

Materials Tro, Qui, Dit, imipramine (Imi), β -NADPH, and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. Liver samples were from Chinese Han nationality adult men, aged 21 - 63 ($37 \pm s 14$) a, who were kidney transplantation donors or patients undergoing hepatic lobectomy with the approval of the local Ethics Committee of Hunan Medical University. All donors were alcoholics. Human liver microsomes were prepared by differential centrifugation^[9]. Microsomal protein were determined with BSA as standard^[10].

In vitro study Microsomes were incubated in a final volume of 500 μL Tris-HCl buffer (100

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mmol·L⁻¹, pH 7.6) using microsomal protein 250 μg. Substrate concentrations for defining the kinetic parameters of Des formation ranged from 1 to 60 μmol·L⁻¹. After therapeutic dosage of Clo, the plasma concentration ranges of Clo and Des are 0.49 – 2.2 and 0.93 – 1.32 μmol·L⁻¹, respectively^[1,11]. Since the drug concentration in liver is always higher than that in plasma, we selected the substrate concentration of 5 μmol·L⁻¹ in inhibition study. Inhibition data points were obtained by incubating Clo 5 μmol·L⁻¹ with Dit 30, Fur 0.01 – 80, Qui 20, Mep 500, Sul 20, and Tro 0.01 – 200 μmol·L⁻¹. Except for Dit (dissolved in water), all inhibitors were dissolved in methanol and evaporated to dryness prior to addition of cofactors and buffer. Fur, Tro, and Dit were respectively incubated (37 °C, 15 min) with microsomes and 300 μL β-NADPH 1 mmol·L⁻¹ containing MgCl₂ 50 mmol·L⁻¹ before the addition of Clo. Clo with and without other inhibitors was preincubated at 37 °C for 5 min. The reaction was initiated by the addition of 300 μL β-NADPH (1 mmol·L⁻¹) containing MgCl₂ (50 mmol·L⁻¹). After incubation at 37 °C for 10 min, the reaction was terminated by adding 100 μL NaOH (6 mol·L⁻¹), followed by the addition of 100 μL internal standard, Imi (36 μmol·L⁻¹). All incubations were performed in duplicate.

Sample analysis Concentrations of Clo and Des were determined by HPLC with Imi as internal standard^[13,14]. A RP-phenyl column (Spherisorb C₆H₅, 250 mm × 4.6 mm ID, 5 μm particle size, China) was used. The wavelength was set at 198 nm. The eluent consisted of 64 % aqueous sodium perchlorate solution (which was prepared by adding NaClO₄ 3 g and 60 % HClO₄ 0.3 – 1 000 mL of redistilled water and adjusted to pH 4.0 with triethylamine) and 36 % acetonitrile, at the flow rate of 1.3 mL·min⁻¹. The relative recoveries of Clo and Des were 96.3 % and 95.1 %, respectively. The absolute recoveries (*n* = 3) were (91.5 ± 4.5) %, (84.3 ± 4.0) % and (93.5 ± 4.5) % for Imi, Des, and Clo, respectively. The intra- and inter-day coefficients of variation were < 7 %. The lower limit of detection was 5 nmol·L⁻¹ with the linear range of 0.05 – 200

μmol·L⁻¹ for both Clo and Des.

Data analysis The kinetic parameters were obtained from the Michaelis-Menten equation. The inhibition data were analyzed by the paired *t* test. IC₅₀ values were obtained by linear regression analysis.

RESULTS

The formation rate of Des was linear with microsome protein ≤ 0.5 g·L⁻¹ and with time ≤ 10 min. A 10-min incubation time and microsomal protein 0.5 g·L⁻¹ were selected.

Des formation increased with Clo concentration before the metabolising enzymes were saturated (Fig 1A). Clo *N*-demethylation

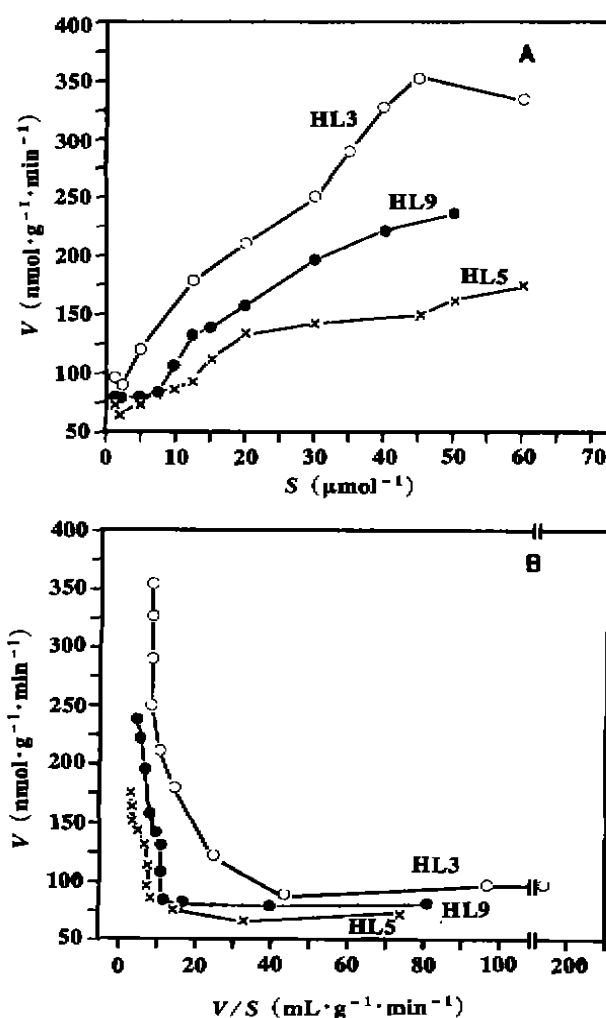


Fig 1. The representative plots of Des-formation rates (Velocity: *V*) vs Clo (substrate: *S*) concentrations in human liver microsomes from HL3, 5, 9 (A) and the corresponding Eadie-Hofstee plots (B).

exhibited an apparently biphasic behavior in the absence of inhibitors (Fig 1B), suggesting that at least two CYP isoforms were involved in the Des formation in human liver microsomes.

Michaelis-Menten equation was used to estimate the affinity constant, K_m , the maximal velocity, V_{max} , and the intrinsic clearance (C_{lim}) defined as V_{max}/K_m , (Tab 1). The interindividual variations reached up to 2.5-, 7.3-, 3.4-, and 1.8-fold for V_{max1} , V_{max1}/K_{m1} , V_{max2} , and V_{max2}/K_{m2} , respectively.

Tab 1. Kinetic parameters of clomipramine *N*-demethylation in human liver microsomes.

Microsomes	K_{m1} $\mu\text{mol}\cdot\text{L}^{-1}$	V_{max1} $\text{nmol}\cdot\text{g}^{-1}\cdot\text{min}^{-1}$	V_{max1}/K_{m1} $\text{L}\cdot\text{g}^{-1}\cdot\text{min}^{-1}$	K_{m2} $\mu\text{mol}\cdot\text{L}^{-1}$	V_{max2} $\text{nmol}\cdot\text{g}^{-1}\cdot\text{min}^{-1}$	V_{max2}/K_{m2} $\text{L}\cdot\text{g}^{-1}\cdot\text{min}^{-1}$
HL3	0.06	104	1.8	22	478	0.021
HL5	0.12	78	0.6	14	210	0.015
HL6	0.15	193	1.3	14	382	0.026
HL7	0.18	113	0.6	48	718	0.015
HL9	0.02	81	4.6	24	355	0.015
\bar{x}	0.11	114	1.8	24	428	0.019
s	0.06	47	1.6	11	188	0.005

At the selected concentration ($5 \mu\text{mol}\cdot\text{L}^{-1}$) of Clo, Tro, Fur, Dit, and Mep produced marked inhibition (paired *t* test, $n = 4 - 5$) on Clo *N*-demethylase activity while Sul and Qui had only slight effects (Fig 2).

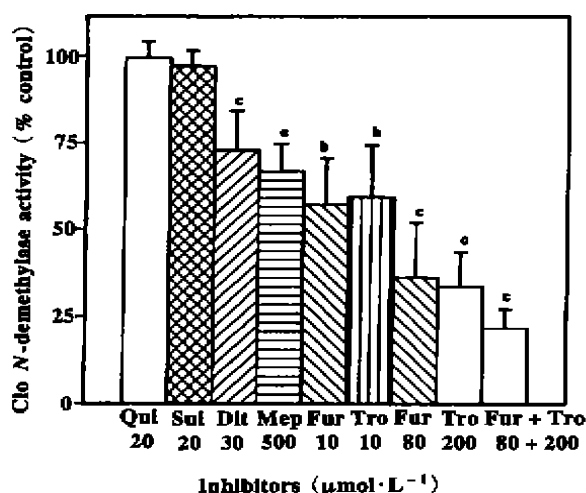


Fig 2. Effects of selective CYP450 inhibitors on the *N*-demethylation of Clo ($5 \mu\text{mol}\cdot\text{L}^{-1}$) in human liver microsomes. $n = 4 - 5$. ^a $P < 0.05$, ^e $P < 0.01$ vs control.

The inhibitory rates ($n = 4 - 5$) produced by Dit 30, Mep 500, Fur 10, Tro 10, Fur 80, and Tro 200 $\mu\text{mol}\cdot\text{L}^{-1}$ were 27.0 %, 32.9 %, 42.8 %, 40.5 %, 63.9 %, and 66.4 %, respectively. The combined inhibitory rate of Tro 200 and Fur 80 $\mu\text{mol}\cdot\text{L}^{-1}$ was up to 78.3 % ($n = 5$). Fur and Tro resulted in a concentration-dependent inhibition on Des formation with IC_{50} value (95 % confidence limits, $n = 5$) of 27.7 (19.1 - 36.3) $\mu\text{mol}\cdot\text{L}^{-1}$ and 42.1 (20.9 - 63.3) $\mu\text{mol}\cdot\text{L}^{-1}$, respectively.

DISCUSSION

The formation of Des was biphasic and existed large interindividual variation in human liver microsomes^[4,14]. The present study showed that Fur and Tro were the most potent inhibitors of Des formation, but their combined inhibitory rate did not exceed 80 %. Thus, CYP1A2, and CYP3A4 play major but not exclusive roles in the Des formation at therapeutic concentration of Clo. This is in accordance with another study *in vitro*^[14]. The large interindividual variations of CYP1A2 and CYP3A4 contents and activities explain partially the interindividual variation in clinical effect of Clo.

Though Dit and Mep were studied at a concentration of 30 $\mu\text{mol}\cdot\text{L}^{-1}$ (the high limit of selectively inhibited concentration) and 500 $\mu\text{mol}\cdot\text{L}^{-1}$ (greatly in excess of K_m), respectively, both produce an inhibitory effect of less than 50 %. CYP2C19 and 2E1 seemed to catalyze the *N*-demethylation of Clo in part. However, since Dit can also inhibit CYP1A2 and 3A4^[15], the minor role of CYP2E1 on Des formation *in vivo* and *in vitro* need to be confirmed. Sul and Qui had no effect on Clo *N*-demethylation, thus the role of CYP2C9/10 and 2D6 might be negligible.

In summary, the *N*-demethylation of Clo was biphasic. This metabolic pathway was mediated mainly by both CYP1A2 and CYP3A4, to a minor extent by CYP2C19 at the near therapeutic concentration of Clo in human liver microsomes.

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氯米帕明在人肝微粒体中的 N-去甲基代谢¹

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关键词 氯米帕明; 细胞色素 P-450; 咪拉茶碱; 醋竹桃霉素; 美芬妥英; 二硫卡钠; 磺胺苯吡唑; 奎尼丁; 肝微粒体 去甲基代谢¹

目的: 研究 CYP450 选择性抑制剂对体外氯米帕明 (Clo) N-去甲基代谢的影响. 方法: 应用米氏方程计算肝微粒体中 Clo N-去甲基代谢的动力学参数, 比较加入抑制剂前后这些参数的改变. 结果: K_{m1} , K_{m2} , V_{max1} , V_{max2} , V_{max1}/K_{m1} 和 V_{max2}/K_{m2} 分别为 (0.11 ± 0.06) , $(24 \pm 11) \mu\text{mol} \cdot \text{L}^{-1}$, (114 ± 47) , $(428 \pm 188) \text{nmol} \cdot \text{g}^{-1} \cdot \text{min}^{-1}$, (1.8 ± 1.6) 和 $(0.019 \pm 0.005) \text{L} \cdot \text{g}^{-1} \cdot \text{min}^{-1}$. 后四个参数的个体差异分别可达 2.5, 7.3, 3.4 和 1.8 倍. 二硫卡钠(Dit)、美芬妥英(Mep)、咪拉茶碱(Fur)、醋竹桃霉素(Tro)和 Fur + Tro 的抑制率分别为 27.0%, 32.9%, 63.9%, 66.4% 和 78.3%. Tro 和 Fur 的 IC_{50} 分别为 42.1 和 27.7 $\mu\text{mol} \cdot \text{L}^{-1}$. 结论: 人肝微粒体中存在高、低亲和力 Clo N-脱甲基酶. 在低浓度下, Clo N-去甲基代谢主要由 CYP3A4 和 CYP1A2 催化, 其次由 CYP2C19 介导.