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In vitro proguanil activation to cycloguanil is mediated by CYP2C19 and CYP3A4 in adult Chinese liver microsomes[1]

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KEY WORDS antimalarials; metabolism; cytochrome P-450 CYP2C19; cytochrome P-450 CYP3A4; enzyme inhibitiors; mephenytoin

ABSTRACT

AIM: To identify the cytochrome P450 isoforms involved in proguanil (PG) activation to cycloguanil (CG) in Chinese liver microsomes. **METHODS**: The kinetics of the CG formation from PG was determined in the liver microsomes of 6 Chinese subjects. Selective chemical inhibitors to various cytochrome P450 isoforms were employed to conduct inhibition experiments. The relationship between the CG formation and *S*-mephenytoin 4′-hydroxylation was analyzed. **RESULTS**: The kinetic behaviors of CG formation were described well by a single-enzyme Michaelis-Menten equation in five livers. The apparent K_m and V_{max} were (82 ± 47) $\mu mol \cdot L^{-1}$ and (8 ± 6) $pmol \cdot min^{-1} \cdot mg^{-1}$ protein, respectively. However, the remaining one displayed a two-enzyme kinetic behavior. Inhibition experiments showed that troleandomycin (100 $\mu mol \cdot L^{-1}$) and diethyldithiocarbamate (100 $\mu mol \cdot L^{-1}$), as potent CYP3A4 and CYP2E1 inhibitors, respectively, reduced the formation rate of CG by 81.1 % and 47.23 %, while quinidine (10 $\mu mol \cdot L^{-1}$), furafylline (20 $\mu mol \cdot L^{-1}$), and sulfaphenazole (10 $\mu mol \cdot L^{-1}$), which were inhibitors towards CYP2D6, 1A2 and 2C9/10, respectively, did not display significant inhibition. At a low PG concentration of 5 $\mu mol \cdot L^{-1}$, the CG formation correlated well with *S*-mephenytoin 4′-hydroxylation ($r = 0.805$, $P < 0.05$). Nevertheless, when a high substrate concentration (500 $\mu mol \cdot L^{-1}$) was used, the correlation coefficient decreased ($r = 0.581$, $P < 0.05$). **CONCLUSION**: The present study indicates that CYP3A4 and CYP2C19 are involved in PG activation to CG in adult Chinese liver microsomes. CYP2C19 played an important role in the clearance of PG at a substrate concentration close to *in vivo* therapeutic concentrations, while CYP3A4 gradually made a dominant contribution with the increase of PG concentration.

INTRODUCTION

Proguanil (PG), an arylbiguanide agent, has been used as an effective prophylactic drug for antimalarial therapy for nearly 50 years[1]. However, its disposition in humans was still not explicit until the end of 1980s[2]. PG is a pro-drug that requires cyclization to its active metabolite cycloguanil (CG) by cytochrome P450 isoforms. *In vivo* studies have documented that PG activation to CG has a large inter-individual variation and cosegregates with the anticonvulsant *S*-mephenytoin (*S*-MP) oxidation mediated by polymorphically expressed CYP2C19[3-6]. In addition, it has been reported that CYP3A as well as CYP2C19 contribute to PG metabolism *in vitro*[7,8]. However, the relative contributions of the two cytochrome P450 isoforms were not differentiated with respect to substrate concentration. A range of substrate concentration that was from 12.5 to 500 $\mu mol \cdot L^{-1}$ was used in these two *in vitro* studies[7,8], which ranges too far from the *in vivo* therapeutic concentrations of 0.2 - 2.9 $\mu mol \cdot L^{-1}$[2]. The kinetic data from the previous *in vitro* studies thus may not well reflect the PG metabolism *in vivo*. This study was designed to identify the CYP450 isoforms involved in PG activation to its active metabolite CG in the liver microsomes of Chinese subjects, and attention was particularly paid to evaluate the relative contributions of CYP2C19 and CYP3A4 at different substrate concentrations.

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MATERIALS AND METHODS

Chemicals PG and CG were kindly provided by Dr Edstein (Army Malaria Research Unit, Australia). *Quinidine* (QUI), *troleandomycin* (TAO), *diethyldithiocarbamate* (DDC), bovine serum albumin (BSA), and NADPH (sodium salt) were purchased from Sigma (Poole, UK). Sulfaphenazole (SUL) was a gift from Ciba-Geigy Ltd (Basel, Switzerland). Furafylline (FUR) was kindly donated by Dr W Pfleiderer (University Konstanz, Germany). *S*-MP and 4′-hydroxymephenytoin were generously donated by Dr GR Wilkinson (Vanderbilt University School of Medicine, Nashville, TN, USA). All other supplies were of the highest grades available from commercial sources.

Human liver specimens and microsome preparation The study protocol was approved by the *Ethnic Committee of Hunan Medical University*. The ten liver specimens used in this study were obtained from patients undergoing hepatectomy in Hunan Tumor Hospital. The range of age for the six patients was from 37 – 51 years. After about 30 g liver tissue was excised from the patient, it was immediately washed 2 – 3 times with ice-cold 0.9 % NaCl and 1.17 % KCl solution (in 0.1 mol·L^{-1}, pH 7.4 phosphate buffer), then quickly frozen in liquid nitrogen for 10 min and transferred into a −80 ℃ ultra-low temperature freezer. Prior to use, all specimens were confirmed as being normal histologically. Liver microsomes were prepared by differential centrifugation[9]. Protein and cytochrome P450 contents were measured by the methods of Lowry[10] and Omura & Sato[11], respectively. BSA was used as the standard in the determination of protein concentration.

***In vitro* incubation conditions and sample preparation** Initial incubation system contained PG (5 – 500 μmol·L^{-1}), $MgCl_2$ (5 mmol·L^{-1}), microsomes (0.5 – 1.5 mg), with the supplement of pH 7.4 phosphate buffer (0.1 mol·L^{-1}) to a volume of 800 μL. Reaction was initiated in a 37 ℃ shaking water bath by the addition of 200 μL NADPH (1 mmol·L^{-1}), sustained for 30 – 80 min and then terminated by adding 1 mL ice-cold methanol. Sample preparations for high pressure liquid chromatography (HPLC) analysis were carried out by the method of Birkett[7] with minor modifications. Here, 8-hydroxyclomipramine (8-OH CMI) was used as the internal standard for assaying PG and CG. After extraction, the organic phase was transferred to another glass tube and evaporated to dryness under a steady stream of nitrogen at 50 ℃. The residues were then dissolved in 50 μL of HPLC mobile phase, and an aliquot of 20 μL was injected into HPLC. All incubations were performed in duplicate. Incubations absent of NADPH or microsomes were used to authenticate whether the formation of CG was dependent on NADPH or microsomes.

Inhibition experiments The incubation conditions were described as above. TAO (100 μmol·L^{-1}), FUR (20 μmol·L^{-1}), DDC (100 μmol·L^{-1}), SUL (10 μmol·L^{-1}), and QUI (10 μmol·L^{-1}) were used as inhibitors towards CYP3A4, 1A2, 2E1, 2C9/10, and 2D6, respectively[12]. The concentrations of the inhibitors were selected according to their respective inhibition constants (K_i or IC_{50}) or inhibition working concentrations[12]. Except QUI (dissolved in water), the other inhibitors were dissolved in methanol which were added to the bottom of incubation-tube in advance and evaporated to dryness under a steady stream of nitrogen at room temperature. In addition, FUR, DDC and TAO were respectively preincubated in the incubation system at 37 ℃ for 15 min before the addition of PG.

Correlation study The experiments were completed within a day. The incubation conditions for *S*-MP (as a CYP2C19 specific probe substrate) metabolism *in vitro* and the quantification of the major metabolite 4′-hydroxymephenytoin were similar to those described by Goldstein[6]. The correlation of PG cyclization at a lower and a high PG concentration (5 and 500 μmol·L^{-1}, respectively) with *S*-MP (200 μmol·L^{-1}) 4′-hydroxylation was studied in different liver microsomes from 10 Chinese.

Chromatographic conditions The HPLC system was composed of an HP 1050 series of Hewlett-Packard instruments with a 20 μL injector loop and a variable wavelength ultraviolet detector. A reversed-phase phenyl column [Spherisorb, 5 μm, (250 ± 5) mm ID, Alltech Co, Dialian, China] was used to separate PG, CG, and 8-OH CMI. The mobile phase consisted of re-distilled water containing 0.5 % triethylamine (adjusted to pH 2.7 by phosphate) and acetonitrile (76∶24, v/v) at a flow rate of 1.0 mL·min^{-1}. The column temperature was kept at 30 ℃ by an air-conditioner, and the eluent was monitored at 238 nm. The sample analysis was completed within approximately 12 min. The identification of CG was confirmed by comparing its retention time with that of CG standard solution. The absolute recoveries for PG, CG, and 8-OH CMI were 84.4 %, 77.6 %, and 83.7 % with variant coefficients

less than 6 %, respectively. The unknown content of CG was determined by comparing peak area with a CG calibration curve made with six CG concentrations at a range of 50 – 2000 nmol·L^{-1}.

Statistical analysis The data were presented as $\bar{x} \pm s$ when appropriate. A P value of <0.05 was considered statistically significant in the correlation analysis. Enzyme kinetics was analyzed by means of non-linear least square curve fitting (Figperfect, ver 5.0, Software Cooperation 1990). The kinetic data were described respectively by the mono- and two-enzyme models of Michaelis-Menten (MM) equations that are presented as followings:

$$V = V_{max} \pm S/(K_m + S) \quad (1)$$

$$V = V_{max1} \pm S/(K_{m1} + S) + V_{max2} \pm S/(K_{m2} + S) \quad (2)$$

Where V is the formation rate of CG, S is the substrate (PG) concentration, and K_m is the substrate concentration at which the formation rate is 50 % of V_{max} which is the maximum formation rate of CG. While K_{m1} and V_{max1}, and K_{m2} and V_{max2} correspond to the high- and low-affinity components of PG cyclizase, respectively, when appropriate. V_{max}/K_m was employed to reflect intrinsic clearance (Cl_{int}) of PG *in vitro*.

RESULTS

In the absence of NADPH or liver microsomes, the amount of CG could not be detected in incubations. The formation rates of CG were linear for the incubation time up to at least 80 min and for microsomal protein concentration up to 1.25 g·L^{-1}, respectively.

Kinetic characteristics of PG cyclization By using a software (Figperfect, ver 5.0, Software cooperation 1990), a non-linear least squares regression was employed to fit the kinetic data from six livers to the models described in methods. We found that the PG cyclizase kinetics was described better with the mono-enzyme model of MM equation than with that of the two-enzyme model in five of the six livers. Thus the former model was used to calculate the kinetic parameters K_m and V_{max} for the five livers. The values are showed in Tab 1. The representative Eadie-Hofstee plot (from HL-13) was shown in Fig 1 (●), which was linear over a PG concentration range of 5 – 500 μmol·L^{-1}. However, the kinetic data from the remaining one (coded HL-4) could be fitted better to the two-enzyme model (Fig 1, ▲). Apparent K_m and V_{max} values for the high- (K_{m1} and V_{max1}) and low-affinity (K_{m2} and V_{max2}) components were 4.4, 81.4 μmol·L^{-1} and 0.31, 4.79 pmol·min^{-1}·mg^{-1} protein, respectively.

Tab 1. Computer derived values of kinetic parameters of PG activation to CG in five Chinese liver specimens according to a mono-enzyme model of Michaelis-Menten equation.

No	V_{max}/pmol·min^{-1}·mg^{-1} protein	K_m/μmol·L^{-1}	V_{max}/K_m (Cl_{int})/μL·min^{-1}·mg^{-1}
HL20	1.7	50	0.034
HL10	16.4	155	0.105
HL11	9.0	39	0.230
HL13	4.7	64	0.073
HL 7	7.8	104	0.075
$\bar{x}$	8	82	1.031
s	6	47	0.075

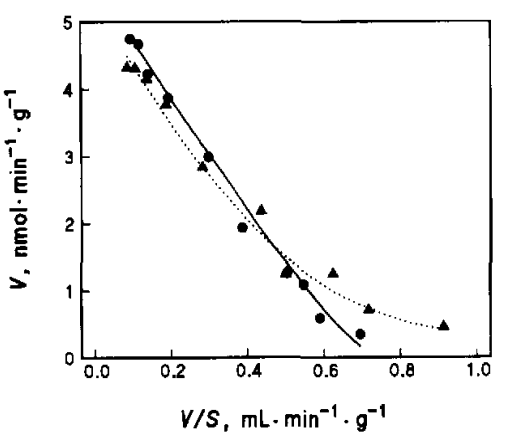

Fig 1. Representative Eadie-Hofstee plots of PG activation to CG with liver microsomes from HL-13 (●) and HL-4 (▲). The Y-axis is the velocity (V) of CG formation, while the X-axis is the ratio of V to substrate concentration (S).

Inhibition experiments The kinetic behavior of the PG activation to CG was further studied in the presence of various inhibitors towards CYP1A2 (FUR), CYPC9/10 (SUL), CYP2D6 (QUI), CYP2E1 (DDC), and CYP3A4 (TAO) with the microsomes from two livers coded HL-4 and HL-11. The effects of the inhibitors on the maximal CG formation rate (V_{max}) are presented in Fig 2. The K_m values for the two livers with the inhibitors were similar to those without the inhibitors (data not shown). TAO (100 μmol·L^{-1}), DDC (100 μmol·L^{-1}) reduced the V_{max} by 81.1 % and 47.23 %, respectively. However, little inhibition was observed with

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FUR, SUL, and QUI (< 14.0 %) (Fig 2). When comparing the CG formation rate in the presence or absence of TAO at 5 μmol·L^{-1} PG, we found TAO only caused a mean 38.5 % reduction. With the increase in PG concentration, the inhibition percentage increased, and finally reached 81.1 % at 500 μmol·L^{-1} PG.

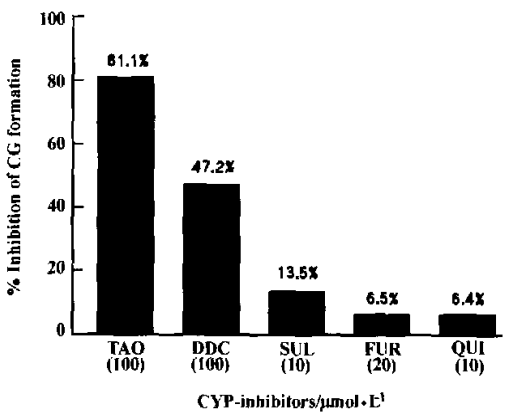

Fig 2. Effects of CYP-inhibitors on the maximal formation rate of PG activation to CG in incubation with liver microsomes from HL-11 and HL-4. The values are mean of the two livers. The CYP-inhibitors used here were troleandomycin (TAO), diethyldithiocarbamate (DDC), sulfaphenazole (SUL), furafylline (FUR), and quindine (QUI). The number in each parenthesis in the figure represents the concentration (μmol/L) of each inhibitor used.

Correlation study Whether CYP2C19 is involved in the activiation of PG to CG in Chinese liver microsomes was evaluated by using correlation analysis (Fig 3). When using 5 μmol·L^{-1} PG, a strong correlation was found between PG cyclization and *S*-MP 4'-hydroxylation activities in 10 livers ($r = 0.805$, $P < 0.05$). However, the extent of the correlation was reduced when using 500 μmol·L^{-1} PG ($r = 0.581$, $P < 0.05$).

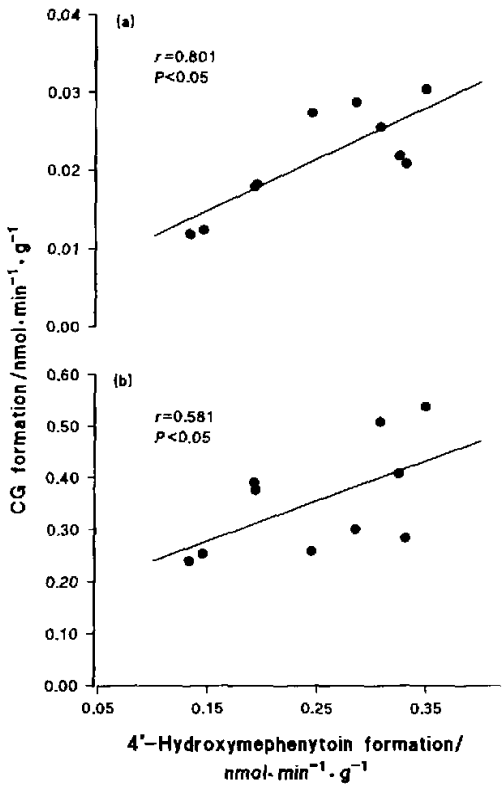

Fig 3. Correlation of PG activation to CG with S-mephenytoin 4'-hydroxylation in the liver microsomes from 10 Chinese. (a) 5 μmol·L^{-1} PG, (b) 500 μmol·L^{-1} PG.

DISCUSSION

To predict the *in vivo* drug pharmacokinetics from the data of drug metabolism studies *in vitro*, it is important to carry out such studies at physiologically or clinically relevant substrate concentrations[9,13]. A wide range of substrate concentrations including those close to *in vivo* therapeutic concentrations increases the chances of detecting the "high affinity" components of the metabolic enzymes of a given drug. Such high-affinity components are often the most clinically relevant among the drug-metabolizing enzymes[9,13]. In the present study, we used a range of PG concentrations from 5 ~ 500 μmol·L^{-1} whose lower limit was close to the C_{max}(0.2 – 2.9 μmol·L^{-1}) of PG in human plasma after the administration of a traditional PG dose (200 mg *po*)[2].

The kinetic parameters K_m, V_{max} and Cl_{int} (V_{max}/K_m) for five of the six livers studied in kinetic experiments were calculated from the mono-enzyme model of MM equation. However, the kinetic behavior of another liver (HL-4) was somewhat different. It better fitted a two-enzyme model of MM equation, and its corresponding Eadie-Hofstee plots were non-linear [Fig 1 (▲)], suggesting that two kinds of CYP450 isoforms, ie, a high-affinity and the other low-affinity component, were involved in the activation of PG to CG in this liver. However, the low-affinity components appear to have a 15 times higher V_{max} than the high-affinity ones. Calculated from the kinetic parameters from HL-4 using the

equation 2 (see methods), the contribution of the high-affinity components to CG formation is negligible at substrate concentrations more than 50 $\mu mol \cdot L^{-1}$. It is thus reasonable to assume that the contribution of high-affinity components to CG formation may be masked by the high-affinity components to a great extent. Such an assumption is a possible explanation for why the two-enzyme model could not be used to fit the kinetic data from the other 5 livers.

We observed that 100 $\mu mol \cdot L^{-1}$ DDC could significantly reduce the formation of CG. However, the involvement of CYP2E1 in PG metabolism needs to be further studied because it has been recently reported that DDC is not a highly selective inhibitor towards CYP2E1[14]. In the present study, CYP3A4-selective inhibitor TAO could cause an 81.1 % inhibition for CG formation at 500 $\mu mol \cdot L^{-1}$ PG but only 38.5 % at 5 $\mu mol \cdot L^{-1}$ PG, suggesting CYP3A4 plays a major role in the activation of PG to CG at high substrate concentrations but a minor role at low substrate concentrations. Furthermore, the present results of correlation study showed that at a low substrate concentration of 5 $\mu mol \cdot L^{-1}$, but not at a high one (500 $\mu mol \cdot L^{-1}$), the activation of PG to CG mainly cosegregated with CYP2C19-mediated *S*-MP 4′-hydroxylation. In combination with kinetic studies, these data from inhibition and correlation studies indicate that CYP2C19 is the high-affinity enzyme for PG cyclization while CYP3A4 is the low-affinity one.

In conclusion, this study shows that both CYP2C19 and CYP3A4 are involved in the activation of PG to CG in adult Chinese liver microsomes. Furthermore, CYP2C19 is the major CYP450 responsible for the reaction at low substrate concentrations close to *in vivo* therapeutic PG, whereas CYP3A4 is the major one at high substrate concentrations and more likely to serve as a kind of physiological "back-up".

REFERENCES

1 Carrington HC, Crowther AF, Davey DG, Levi AA, Rose FL. A metabolite of "Paludrine" with high antimalarial activity. Nature 1951; 68: 1080 – 6.

2 Wattanagoon Y, Taylor RB, Moody RR, Ochekpe NA, Looareesuwan S, White NJ. Single dose pharmacokinetics of proguanil and its metabolites in healthy subjects. Br J Clin Pharmacol 1987; 24: 775 – 80.

3 Ward SA, Watkins WM, Mberu E, Saunders JE, Koech DK, Gilles HM, *et al*. Inter-subject variability in the metabolism of proguanil to the active metabolite cycloguanil in man. Br J Clin Pharmacol 1989; 27: 781 – 7.

4 Basci NE, Bozkurt A, Kortunay S, Isimer A, Sayal A, Kayaalp SO. Proguanil metabolism in relation to *S*-mephenytoin oxidation in a Turkish population. Br J Clin Pharmacol 1996; 42: 771 – 3.

5 Setiabudy R, Kusaka M, Chiba K, Darmansjah I, Ishizaki T. Metabolic disposition of proguanil in extensive and poor metabolisers of *S*-mephenytoin 4′-hydroxylation recruited from an Indonesian population. Br J Clin Pharmacol 1995; 39: 297 – 303.

6 Goldstein JA, Faletto MB, Romkes-Sparks M, Sullivan T, Raucy JL, Kitareewan S, *et al*. Evidence for a role for 2C19 in metabolism of *S*-mephenytoin in humans. Biochemistry 1994; 33: 1743 – 52.

7 Helaby NA, Ward SA, Howells RE, Breckenridge AM. *In vitro* metabolism of the biguanide antimalarials in human liver microsomes: evidence for a role of the mephenytoin hydroxylase (P450MP) enzyme. Br J Clin Pharmacol 1990; 30: 287 – 91.

8 Birkett DJ, Rees D, Andersson T, Gonzalez FJ, Miners JO, Vernose ME. *In vitro* proguanil activation to cycloguanil by human liver microsomes is mediated by CYP3A isoforms as well as by *S*-mephenytoin hyddroxylase. Br J Clin Pharmacol 1994; 37: 413 – 20.

9 Shu Y, Xu ZH, Xie HG, Zhu RH, Zhao JP, Zhou HH. Enzyme kinetic analysis and inhibition of amitriptyline *N*-demethylation in human liver microsomes *in vitro*. Chin J Pharmacol Toxicol 1998; 12: 260 – 5.

10 Lowry OH, Roseborough WJ, Farr AL, Randall RJ. Protein measurement with Folin reagent. J Biol Chem 1951; 193: 265 – 75.

11 Omura T, Sato R. The Carbon-monoxide binding pigment of liver microsomes. J Biol Chem 1964; 239: 2370 – 8.

12 Newton DJ, Wang RW, Lu AYH. Cytochrome P450 inhibitors: evaluation of specificities in the *in vitro* metabolism of therapeutic agents by human liver microsomes. Drug Metab Dispos 1995; 23: 154 – 8.

13 Kato R, Yamazoe Y. The importance of substrate concentration in determining cytochrome P450 therapeutically relevant *in vivo*. Pharmacogenetics 1994; 4: 359 – 62.

14 Eagling VA, Tjia JF, Back DJ. Differential selectivity of cytochrome P450 inhibitors against probe substrates in human and rat liver microsomes. Br J Clin Pharmcol 1998; 45: 107 – 14.

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体外中国成人肝微粒体中氯胍活化为氯胍三嗪由 CYP2C19 和 CYP3A4 介导[1]

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关键词　抗疟药；代谢；细胞色素 P-450 CYP2C19；细胞色素 P-450 CYP3A4；酶抑制剂；美芬妥英

目的：鉴定中国成人肝微粒体中介导氯胍（PG）活化为氯胍三嗪（CG）的细胞色素 P450（CYP450）. **方法**：分析中国成人（$n=6$）肝微粒体中 PG 活化为 CG 的酶促动力学，各种 CYP450 抑制剂对该代谢的作用及其与 *S*-美芬妥英 4′-羟化的关系. **结果**：6 个标本中，除一个外（两酶米氏模型），PG 活化为 CG 的酶促动力学符合米氏一酶模型；CYP3A4 和 CYP2E1 的选择性抑制剂醋竹桃霉素（81.1 %）和二乙二硫基苯甲酸（47.23 %）可抑制 CG 生成，其它抑制剂没有明显作用；在低 PG 浓度时，PG 的环化与 *S*-美芬妥英 4′-羟化显著相关（$r=0.805$，$P<0.05$），高浓度时相关性明显减小. **结论**：在中国成人肝微粒体中 CYP2C19 和 CYP3A4 参与了 PG 活化为 CG.

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