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Effects of CYP2C19 genotype and CYP2C9 on fluoxetine *N*-demethylation in human liver microsomes[1]

LIU Zhao-Qian, SHU Yan, HUANG Song-Lin, WANG Lian-Sheng, HE Nan, ZHOU Hong-Hao[2]
(*Pharmacogenetics Research Institute, Hunan Medical University, Changsha 410078, China*)

KEY WORDS fluoxetine; pharmacokinetics; liver microsomes; cytochrome P-450 CYP2C19; cytochrome P-450 CYP2C9

ABSTRACT

AIM: The present study was designed to define the kinetic behavior of fluoxetine *N*-demethylation in human liver microsomes and to identify the isoforms of cytochrome P-450 (CYP) involved in this metabolic pathway. **METHODS**: The kinetics of the formation of norfluoxetine was determined in human liver microsomes from six genotyped CYP2C19 extensive metabolizers (EM). The correlation studies between the fluoxetine *N*-demethylase activity and various CYP enzyme activities were performed. Selective inhibitors or chemical probes of various cytochrome P-450 isoforms were also employed. **RESULTS**: The kinetics of norfluoxetine formation in all liver microsomes were fitted by a single-enzyme Michaelis-Menten equation (mean K_m = 32 μmol/L ± 7 μmol/L). Significant correlations were found between *N*-demethylation of fluoxetine at both 25 μmol/L and 100 μmol/L and 3-hydroxylation of tolbutamide at 250 μmol/L ($r_1 = 0.821$, $P_1 = 0.001$; $r_2 = 0.668$, $P_2 = 0.013$), respectively, and *S*-mephenytoin 4′-hydroxylase activity ($r = 0.717$, $P = 0.006$) at high substrate concentration of 100 μmol/L. *S*-mephenytoin (SMP) (a CYP2C19 substrate) at high concentration and sulfaphenazole (SUL) (a selective inhibitor of CYP2C9) substantially inhibited norfluoxetine formation. The reaction was minimally inhibited by coincubation with chemical probe, inhibitor of CYP3A4 (triacetyloleandomycin, TAO). The inhibition of fluoxetine *N*-demethylation at high substrate concentration (100 μmol/L) was greater in PM livers than in EM livers (73 % *vs* 45 %, $P < 0.01$) when the microsomes were precoincubated with SUL plus TAO. **CONCLUSION**: Cytochrome P-450 CYP2C9 is likely to be a major CYP isoform catalyzing fluoxetine *N*-demethylation in human liver microsomes at a substrate concentration close to the therapeutic level, while polymorphic CYP2C19 may play a more important role in this metabolic pathway at high substrate concentration.

INTRODUCTION

Fluoxetine (FLU) is a potent and selective serotonin reuptake inhibitor (SSRI) in the central nervous system and is extensively used to treat depression and obsessive-compulsive behavior[1,2]. Previous studies have found that SSRI differently inhibit the activity of various cytochrome P-450 (CYP) isoforms, including CYP1A2, CYP2D6, CYP2C19, CYP2C9, and CYP3A4[3-5]. Fluoxetine and its principal metabolite, norfluoxetine (*N*-FLU), are both potent inhibitors of CYP2D6, 3A4, and 2C19[6]. From a clinical point of view, an important aspect of FLU is its ability to cause dangerous interactions when coadministered with other drugs metabolized by these enzymes. Particularly, this may be a problem even after discontinuation of FLU because both parent compound and in particular *N*-FLU have very long half-lives (2 and 7 days) after a single dose and even longer after repeated dosing. Therefore, the identification of the enzymes responsible for the metabolism of FLU should allow physicians to anticipate and avoid unwanted drug interactions.

FLU is extensively metabolized by the hepatic cytochrome P-450 enzyme and less than 2.5% of the drug is recovered unchanged in human urine[7]. FLU in humans mainly undergoes *N*-demethylation leading to the formation of the active metabolite norfluoxetine[1,2]. However, little is known with regard to the isoforms of cytochromes P-450 responsible for the metabolism of

[1] Project supported by China Medical Association 92-568 and 99-697.
[2] Correspondence to Prof ZHOU Hong-Hao.
Phn 86-731-448-7233. Fax 86-731-447-1339.
E-mail hhzhou@public.cs.hn.cn
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FLU. Recently, von Moltke *et al* have reported that CYP2C9 appears to be the principal human cytochrome mediating FLU *N*-demethylation and CYP2C19 and CYP3A4 may make a small contribution[8]. While, an *in vivo* study has shown that CYP2D6 plays an important role in the disposition of FLU and CYP2D6 contributes significantly to the *N*-demethylation of FLU[9]. Thus, there were some discrepancies on identification of CYP isoforms responsible for the *N*-demethylation of FLU among these studies.

Considering the involvement of multi-enzymes and the different contributions of various CYP isoforms in the *N*-demethylation of FLU, we made preliminary evaluation regarding any correlation between the FLU *N*-demethylase and the various CYP enzyme activities. To test the role of CYP2C19 in the *N*-demethylation of FLU, we have studied the inhibitory effect using different genotyped human liver microsomes from four extensive metabolizers (EM) and three poor metabolizers (PM) with respect to CYP2C19. Various selective chemical inhibitors were also utilized to identify the isoforms of CYP involved in fluoxetine *N*-demethylation.

MATERIALS AND METHODS

Chemicals FLU and *N*-FLU, both as hydrochloride salts, were supplied by Research Biochemicals International (Natick, USA). Nortriptyline, quinidine (QUI), triacetyloleandomycin (TAO), diethyldithiocarbamate (DDC), $NADP^+$, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase were purchased from Sigma Chemical Co (St Louis, USA). Sulfaphenazole (SUL) and ketoconazole (Ket) were gifts from Ciba-Geigy Ltd (Basel, Switzerland) and Janssen Research Foundation (Beerse, Belgium), respectively. Furafylline (FUR) and *S*-mephenytoin (SMP) were kindly donated by Dr W PFLEIDERER (Universität Konstanz, Germany). Acetonitrile of HPLC grade and double distilled water were used for HPLC with UV detector. All other chemicals were of AR grade.

Preparation of human liver microsomes Adult human liver tissue from renal transplant donors without known liver disease and patients who had undergone partial hepatectomy were collected in our liver bank. The collection and utilization of human liver tissues were approved by the Ethics Committee of Hunan Medical University. Candidate patients for liver sample collection were those who did not suffer from acute or chronic hepatitis or cirrhosis, and took no medications known to induce or inhibit CYP activity. Portions of surgical liver 'waste tissue' distant from disease-affected regions and which appeared visually normal were collected. The collection process of liver tissue and its morphologic and biochemical characterization were followed as described elsewhere[10]. Microsomes were prepared by differential centrifugation[10] and stored at −80 ℃ until required. Microsomal protein concentration was determined by the method of Lowry *et al*[11].

Donors were genotyped with respect to CYP2C19 from whole blood or liver tissue according to the method of de Morais *et al*[12]. All the PM were homozygous for the m1 mutation of CYP2C19.

***In vitro* incubation conditions** The incubation mixture contained 0.1 g/L of human liver microsomal protein, potassium phosphate buffer 0.1 mol/L (pH 7.4), reduced NADP (NADPH)-generating system and various concentrations of FLU with or without inhibitors in a final volume of 500 μL. The enzyme reaction was initiated by adding 115 μL NADPH-generating system consisting of NADP 1 mmol/L, glucose-6-phosphate 10 mmol/L, glucose-6-phosphate dehydrogenase 2 kU/L, $MgCl_2$ 10 mmol/L. After incubation at 37 ℃ in a shaking water bath for 75 min, the reaction was stopped by cooling on ice and adding 100 μL acetonitrile. Preliminary experiments showed that the formation of *N*-demethylation was linear with respect to time over 75 min and with respect to microsomal protein concentration (0.1 − 0.2 g/L) at 37 ℃. Accordingly, the incubation time of 75 min and the microsomal protein concentration of 0.1 g/L were chosen in the present study.

Assay and kinetics of *N*-FLU formation *N*-FLU was determined by HPLC with UV detector at 226 nm based on the method developed by Meineke *et al*[13]. Only a one-step extraction with *n*-hexane and acetonitrile (volume percentage, $\varphi = 98:2$) was used, and the internal standard was nortriptyline. The CYP inhibitors used in the study did not produce an interfering peak.

In the kinetic experiments, ten concentrations of FLU (2.5 − 250 μmol/L) were incubated with human liver microsomes from six EM of CYP2C19. A single-enzyme model with Michaelis-Menten was fitted to the data of kinetic experiments by the following equation using non-linear least square regression analysis. The most appropriate model was selected on the basis of the dispersion of residuals and whether an *F*-test showed a significant reduction ($P < 0.05$) in the residual sums of square (Figperfect, Version 5.0)

$V = V_{max} \times S/(K_m + S)$

Correlation studies To assess which of the CYP isoforms may be a major CYP enzyme responsible for *N*-demethylation of FLU, three different concentrations (2.5 μmol/L, 25 μmol/L, 100 μmol/L) of FLU and thirteen human liver microsomes from ten EM and three PM with respect to CYP2C19 were used and any correlation between the FLU *N*-demethylase activity and *S*-mephenytoin 4′-hydroxylase (CYP2C19) activity, tolbutamide 3-hydroxylase (CYP2C9) activity, debrisoquine 4-hydroxylase (CYP2D6) activity, and phenacetin *O*-deethylase (CYP1A2) activity was observed.

Inhibition studies According to the results of the correlation studies, inhibitory studies were performed using fixed substrates concentration of 25 μmol/L. Varying concentrations of a number of possible inhibitors or probe substrates used were SUL (CYP2C9 inhibitor), SMP (CYP2C19 inhibitor), FUR (CYP1A2 inhibitor), QUI (CYP2D6 inhibitor), DDC (CYP2E1 inhibitor), and TAO and Ket (CYP3A4 inhibitor). All inhibitors or probe substrates were coincubated with microsomal preparations from four EM of CYP2C19.

To assess further the effect of CYP2C19 on FLU *N*-demethylation, the rate of formation of *N*-FLU was determined in four EM and three PM liver microsomal preparations at 100 μmol/L substrate concentration of FLU after the microsomal preparations were precoincubated with SUL plus TAO.

The FLU *N*-demethylation activity in the presence of inhibitors or probe substrates was expressed as percentage of the corresponding control values. Results are expressed as $\bar{x} \pm s$ throughout the text. A one-way *t* test for unpaired and paired data was used to determine the significance of differences in the inhibitory effect of chemical inhibitors and probe substrates, with $P < 0.05$ as the minimal level of significance.

RESULTS

Kinetics for *N*-FLU formation Substrate concentration *vs* velocity for the formation of *N*-FLU in human liver microsomes from an EM was shown in Fig 1. Similar plots were obtained with the other five EM microsomal preparations. We found that the kinetics of *N*-FLU formation in six EM of CYP2C19 followed a single-enzyme Michaelis-Menten equation. The overall mean K_m value was (32 ± 7) μmol/L.

SMP (600 μmol/L) and SUL (25 μmol/L) caused

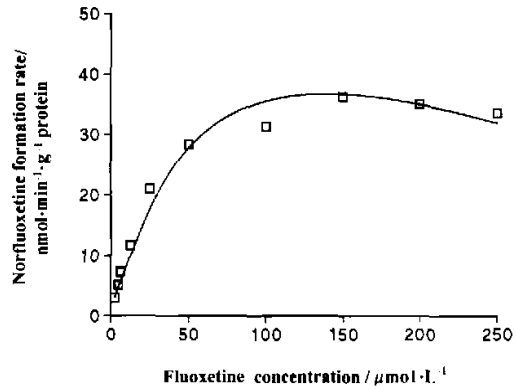

Fig 1. Typical substrate *vs* velocity plot for the formation of *N*-FLU in human liver microsomes from an EM with respect to CYP2C19. The values were the means of duplicate incubations.

a maximum of 18 % and 63 % reduction compared to the control value in the formation of *N*-FLU, respectively, at a substrate concentration of 25 μmol/L, and a maximum of 56 % and 43 % inhibition of *N*-FLU formation was found by SMP (600 μmol/L) and SUL (25 μmol/L), respectively, at a substrate concentration of 100 μmol/L (Fig 2). Although SMP is relatively a weak inhibitor of the *N*-FLU formation, it showed greater activity at high (100 μmol/L) substrate concentration than at the low (25 μmol/L) substrate concentration when 600 μmol/L of SMP was added. TAO (5 μmol/L) and Ket (1.0 μmol/L) reduced the velocity of *N*-FLU formation by 20 % at the substrate concentration of 25 μmol/L, while TAO did not inhibit this metabolic pathway at a substrate concentration of 100 μmol/L. FUR, QUI, and DDC inhibited this reaction to a minor extent.

Correlation studies The formation of *N*-FLU at substrate concentration of 25 μmol/L and 100 μmol/L showed close correlation with 3-hydroxylation of tolbutamide (250 μmol/L) ($r_1 = 0.821$, $P_1 = 0.001$; $r_2 = 0.668$, $P_2 = 0.013$) (Fig 3a), it also showed significant correlation with SMP (500 μmol/L) 4′-hydroxylase activity ($r = 0.717$, $P = 0.006$) at a substrate concentration of 100 μmol/L (Fig 3b). No significant correlation with *O*-deethylation of phenacetine at 250 μmol/L ($r_1 = 0.518$, $P_1 = 0.700$; $r_2 = 0.138$, $P_2 = 0.652$; $r_3 = -0.173$, $P_3 = 0.571$), debrisoquine (250 μmol/L) 4-hydroxylation ($r_1 = -0.119$, $P_1 = 0.712$; $r_2 = 0.350$, $P_2 = 0.241$; $r_3 = 0.210$, $P_3 = 0.491$) at the substrate concentration of 2.5 μmol/L, 25 μmol/L, and 100 μmol/L, respectively. In addition, we found that the

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N-FLU formation had no significant correlation with SMP 4′-hydroxylase activity ($r_1 = 0.278$, $P_1 = 0.358$; $r_2 = 0.537$, $P_2 = 0.059$) at the substrate concentration of 2.5 μmol/L and 25 μmol/L, respectively.

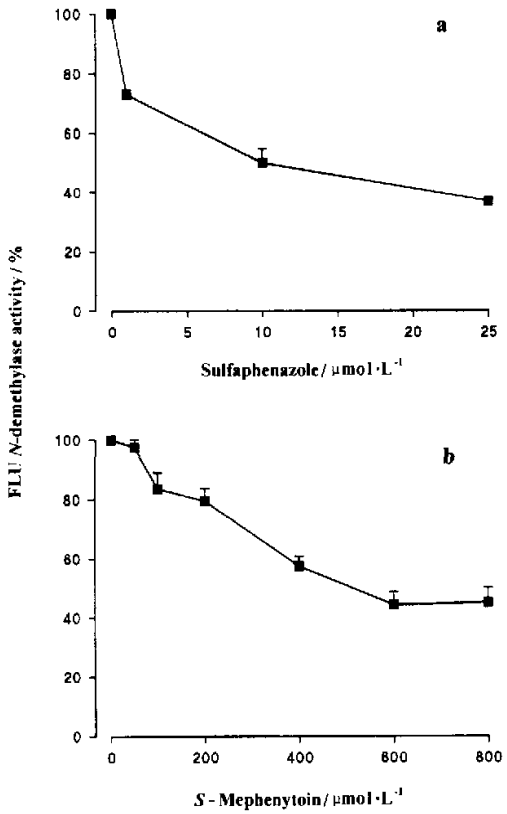

Fig 2. Effects of sulfaphenazole (Fig 2a) and *S*-mephenytoin (Fig 2b) on the formation of norfluoxetine in human liver microsomes from EM. The substrate (fluoxetine) concentration used in Fig 2(a) and (b) was 25 μmol/L and 100 μmol/L, respectively. $n = 4$. $\bar{x} \pm s$.

Effects of polymorphic CYP2C19 on *N*-FLU formation We found that SUL (25 μmol/L) plus TAO (25 μmol/L) produced a maximal inhibition of 73 % and 45 % in the formation of *N*-FLU in human liver microsomes from three genotyped CYP2C19 PM and four EM, respectively. The inhibitory effect on *N*-FLU formation in human liver microsomes from PM with respect to CYP2C19 was greater than that in EM ($P < 0.01$) at a substrate concentration of 100 μmol/L (Fig 4).

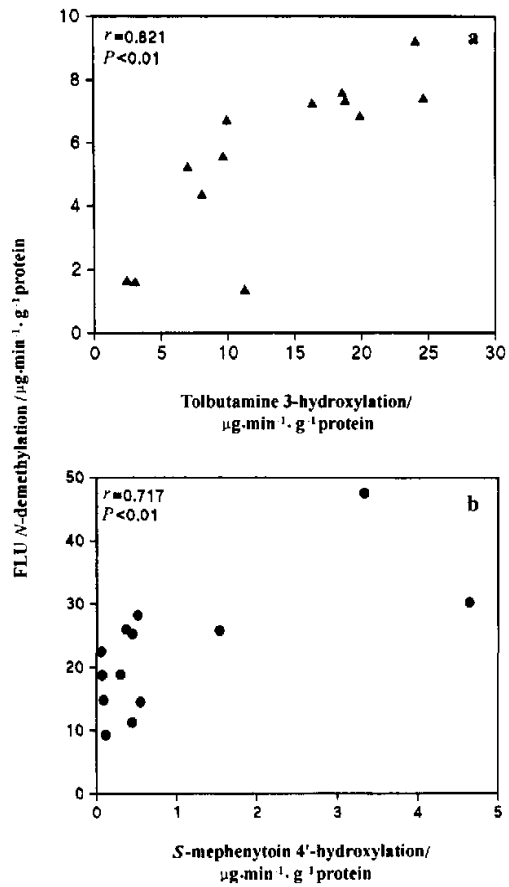

Fig 3. Correlations between fluoxetine *N*-demethylase activity and tolbutamide 3-hydroxylase activity (Fig 3a) and *S*-mephenytoin 4′-hydroxylase activity (Fig 3b) in human liver microsomes from ten EM and three PM of CYP2C19. The substrate concentration used in Fig 3 (a) and (b) was fluoxetine 25 μmol/L and 100 μmol/L, respectively.

DISCUSSION

We observed a monophasic-enzyme kinetics for the formation of *N*-FLU from FLU in human liver microsomes from genotyped PM and EM with respect to CYP2C19. These data indicate clearly that the formation of *N*-FLU was consistent with a single-enzyme Michaelis-Menten kinetics, with a mean K_m of (32 ± 7) μmol/L. Sulfaphenazole (25 μmol/L), a selective and potent inhibitor of CYP2C9[14] inhibited the *N*-demethylation of FLU by up to 63 % at substrate concentration of 25 μmol/L and 43 % at a substrate concentration of 100 μmol/L, indicating the involvement of CYP2C9. The

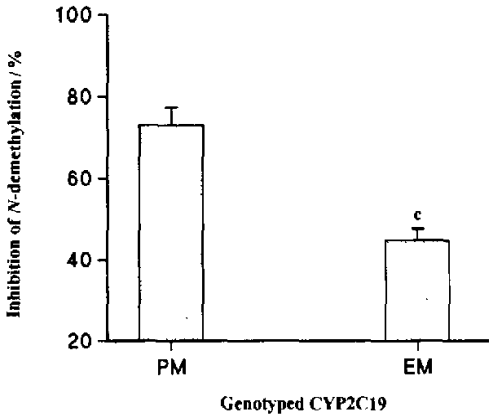

Fig 4. The inhibitory effects of SUL (25 μmol/L) plus TAO (25 μmol/L) on the formation of norfluoxetine in human liver microsomes from four EM and three PM of CYP2C19. The values are given as mean inhibition percentage. $\bar{x} \pm s$. $^cP < 0.01$ *vs* PM.

occurrence of significant correlation between the formation of *N*-FLU and 3-hydroxylation of tolbutamide also supports the involvement of CYP2C9. However, the addition of SUL (25 μmol/L) plus TAO (25 μmol/L) only resulted in a maximal inhibition of 45 % for *N*-FLU formation in human liver microsomes from four EM at a substrate concentration of 100 μmol/L. These data indicate that CYP2C9 may be a major CYP isoform catalyzing FLU *N*-demethylation at a relatively low substrate concentration, and has only a small contribution at high substrate concentration.

S-mephenytoin, a probe substrate of CYP2C19, inhibited substantially more than 50 % activity of FLU *N*-demethylation at high substrate concentration of 100 μmol/L). The foundation of significant correlation between the formation of *N*-FLU and SMP 4′-hydroxylase activity at high substrate concentration of 100 μmol/L, and the inhibitory effect of significant difference in the formation of *N*-FLU in PM and EM (73 % *vs* 45 %, $P < 0.01$) further shows that CYP2C19 is a major CYP isoform responsible for the *N*-demethylation of FLU at high substrate concentration. These data indicate that polymorphic CYP2C19 may play an imporatnt role in the *N*-demethylation of FLU in human liver microsomes. Findings from the present study could in part explain that the genotyped polymorphism of CYP2C19 is likely to be one of the major factors causing the interindividual difference in the steady-state plasma levels of FLU and FLU metabolism.

Except for CYP2C9 and CYP2C19, CYP3A4 also inhibited slightly the formation of *N*-FLU. The present study shows that TAO and Ket at lower concentration of 1.0 μmol/L, a concentration at which Ket is a relatively specific CYP3A inhibitor, caused an approximately 20 % inhibition of *N*-FLU formation at a substrate concentration of 25 μmol/L, while TAO inhibited this reaction to a minor extent at a substrate concentration of 100 μmol/L, and that CYP1A2, 2D6, and 2E1 are not involved. Recently, von Moltke reported that CYP2C9 appeared to be a principal human CYP enzyme responsible for FLU *N*-demethylation and CYP2C19 may make a further small contribution in human liver microsomes at a substrate concentration of 100 μmol/L[8]. However, the present study shows that CYP2C9 and CYP2C19 cause a substantial inhibition in the *N*-demethylation of FLU at a relatively low substrate concentration (25 μmol/L) and at high substrate concentration (100 μmol/L), respectively.

In summary, we conclude that CYP2C9 is likely to be a major CYP enzyme catalyzing FLU *N*-demethylation in human liver microsomes at a substrate concentration close to the therapeutic level, and that polymorphic CYP2C19 may play a more important role at high substrate concentration in this metabolic pathway.

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CYP2C19 基因型和 CYP2C9 对人肝微粒体中氟西汀 *N*-去甲基代谢的影响[1]

刘昭前，舒 焱，黄松林，王连生，何 楠，周宏灏[2]（湖南医科大学遗传药理研究所，长沙 410078，中国）

关键词 氟西汀；药物动力学；肝微粒体；细胞色素 P-450 CYP2C19；细胞色素 P-450 CYP2C9

目的：本实验旨在研究 CYP2C19 基因型人肝微粒体中氟西汀 *N*-去甲基代谢的酶促动力学特点并鉴定参与此代谢途径的细胞色素 P-450 酶. **方法**：测定基因型 CYP2C19 肝微粒体中去甲氟西汀形成的酶促动力学. 鉴定氟西汀 *N*-去甲基酶活性与细胞色素 P-450 2C9，2C19，1A2 和 2D6 酶活性的相关性，同时应用各种细胞色素 P-450 酶的选择性抑制剂和化学探针进行抑制实验，从而确定参与氟西汀 *N*-去甲基代谢的细胞色素 P-450 酶. **结果**：去甲氟西汀生成的酶促动力学数据符合单酶模型，并具有 Michaelis-Menten 动力学特征. 当底物浓度为氟西汀 25 μmol/L和 100 μmol/L 时，去甲氟西汀（*N*-FLU）的生成率分别与甲磺丁脲 3-羟化酶活性显著相关（$r_1 = 0.821$，$P_1 = 0.001$；$r_2 = 0.668$，$P_2 = 0.013$），当底物浓度为氟西汀 100 μmol/L 时，*N*-FLU 的生成率与 *S*-美芬妥因 4′-羟化酶活性显著相关（$r = 0.717$，$P = 0.006$）. PM 肝微粒中磺胺苯吡唑和醋竹桃霉素对氟西汀 *N*-去甲基代谢的抑制作用显著大于 EM（73 % *vs* 45 %，$P < 0.01$）. **结论**：在生理底物浓度下，CYP2C9 是催化人肝微粒体中氟西汀 *N*-去甲基代谢的主要 CYP-450 酶；而高底物浓度时，以 CYP2C19 的作用为主.

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