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ORIGINAL ARTICLE

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Genotype-sensitive reversible and time-dependent CYP2D6 inhibition in human liver microsomes

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Abstract

Cytochrome P450 (CYP) 2D6 metabolizes a wide range of xenobiotics and is characterized by a huge interindividual variability. A recent clinical study highlighted differential magnitude of CYP inhibition as a function of *CYP2D6* genotype. The aim of this study was to investigate the effect of *CYP2D6* genotype on the inhibition of dextromethorphan O-demethylation by duloxetine and paroxetine in human liver microsomes (HLMs). The study focused on genotypes defined by the combination of two fully functional alleles (activity score 2, AS 2, n = 6), of one fully functional and one reduced allele (activity score 1.5, AS 1.5, n = 4) and of one fully functional and one non-functional allele (activity score 1, AS 1, n = 6), which all predict extensive metabolizer phenotype. Kinetic experiments showed that maximal reaction velocity was affected by *CYP2D6* genotype, with a decrease in 33% of V_{max} in AS 1 HLMs compared to AS 2 ($P = 0.06$). No difference in inhibition parameters K_i, K_I and k_{inact} was observed neither with the competitive inhibitor duloxetine nor with the time-dependent inhibitor paroxetine. Among the genotypes tested, we found no difference in absolute CYP2D6 microsomal levels with ELISA immunoquantification. Therefore, our results suggest that genotype-sensitive magnitude of drug-drug interactions recently observed in vivo is likely to be due to differential amounts of functional enzymes at the microsomal level rather than to a difference in inhibition potencies across genotypes, which motivates for further quantitative proteomic investigations of functional and variant CYP2D6 alleles.

KEYWORDS

CYP2D6, drug-drug interaction, genetic polymorphisms, human liver microsomes, in vitro-in vivo extrapolation

1 | INTRODUCTION

The pharmacological response of one individual to its treatment is the result of two components: its disposition in the body (pharmacokinetics) and its interactions with the therapeutic target (pharmacodynamics). Pharmacokinetics include different stages: absorption of the drug into the systemic circulation, tissue distribution, metabolism, elimination and transport. Drug-drug interactions (DDIs) occur when two or more drugs interact with each other and may potentially lead to clinical consequences such as adverse drug reactions or lack of therapeutic effect. In a recent meta-analysis, the prevalence of potential unwanted DDIs was estimated to be 33% in general patients and 67% in intensive care patients during their hospital stay.[1] Cytochromes P450 (CYPs) constitute the main enzyme family responsible for oxidative drug metabolism and are highly relevant in the study of drug disposition due to their high variability in terms of expression and activity, which is mainly caused by genetic polymorphisms (especially for

2B6, 2C9, 2C19 and CYP2D6) and environmental factors such as DDIs. Despite its relatively poor abundance in the liver (<2%), CYP2D6 metabolizes a wide range of drugs, including opioid analgesics, antipsychotic, antidepressant and cardiovascular drugs.[2] A huge variability in the activity of CYP2D6 has been observed in the general population[3] and is mainly explained by genetic polymorphisms and DDIs. More than one hundred alleles have been identified and described so far.[4] Relative to the wild-type *1 *CYP2D6* allele, variant alleles may lead to a reduced, normal or even increased activity. According to the Clinical Pharmacogenetics Implementation Consortium (CPIC), various combinations of alleles predict an extensive metabolizer (EM) phenotype: two functional alleles, two reduced function alleles or one functional with one non-functional or reduced function allele.[5]

While the impact of genetic polymorphism and DDI separately has been largely investigated, only little is known regarding the effect of genetic polymorphism on the extent of DDIs, which could explain the high interindividual variability in the extent of DDIs.[6] Indeed, several in vivo studies showed differential magnitudes of DDIs in EM, IM and UM subjects.[7-10] Poor metabolizers (PM) carrying two non-functional alleles of CYP2D6 are not considered prone to DDIs involving this enzyme, because of a null enzymatic activity at baseline. In contrast, UMs of CYP2D6 are expected to respond more prominently to DDIs because of higher baseline levels of CYP2D6. We recently highlighted different magnitudes of DDIs in genetically predicted EM healthy volunteers carrying either two fully functional alleles (*1, *2, *35) or one non-functional allele (*3-8) in association with a fully functional allele.[11]

The aim of the present work was to further assess the impact of reduced function (*41) and non-functional alleles on DDIs involving CYP2D6 in vitro using genotyped human liver microsomes (HLMs) in order to explain the observed differences in magnitude of DDIs in vivo. The study focused on genotypes defined by the combination of two fully functional alleles (activity score 2, AS 2), of one fully functional with one reduced allele (activity score 1.5, AS 1.5) and of one fully functional with one non-functional allele (activity score 1, AS 1).

2 | MATERIALS AND METHODS

2.1 | Chemicals

Dextromethorphan and its metabolite dextrorphan (purity >99%) were provided by the Pharmacy of Geneva University Hospitals and by Hoffman-La Roche (Basel, Switzerland), respectively, stored at −20°C as 1 mg/mL solution in methanol. Paroxetine, duloxetine, O-desmethyltramadol-D6, nicotinamide adenine dinucleotide phosphate (NADP), isocitric acid, magnesium chloride ($MgCl_2$), isocitrate deshydrogenase and potassium hydroxide were purchased from Sigma-Aldrich (Buchs, Switzerland). Potassium dihydrogenophosphate was purchased from BDH laboratory Supplies (Poole, BH15 1TD, UK). Acetonitrile, methanol and formic acid (ULC/MS quality) were purchased from Biosolve (Dieuze, France). Ultra-pure water was obtained from a Milli-Q-RG purification system (Millipore, Bedford, MA, USA).

2.2 | Genotyped HLMs

Genotyped HLMs from 16 different donors were provided from Xenotech (Kansas City, KS, USA). According to the furnisher, *CYP2D6* genotype was assessed by target SNP Taqman® assays screening for the following main allelic variants: *2, *3, *4, *5, *6, *9, *10, *17, *29, *40, *41 and *1, *2 and *4 gene duplications. According to available data,[12] these allelic variants allow to identify 99.2% of reduced function, non-functional or duplicated CYP2D6 proteins.

2.3 | Dextromethorphan O-demethylation velocity

Various concentrations of dextromethorphan ranging from 0.5 to 100 µmol/L were incubated with genotyped human liver microsomes (final protein concentration: 0.5 mg/mL) in phosphate buffer 0.1 mol/L pH 7.4. Following 3-min. preincubation in a 37°-warmed water bath, the reaction was initiated by addition of a NADPH generating system (NADP 4 mmol/L, isocitrate 15 mmol/L, $MgCl_2$ 12.5 mmol/L and isocitrate dehydrogenase 1.5 IU/mL in reaction buffer). Incubations were performed for 20 minutes. (linear condition, as assessed in a preliminary experiment) at 37°C. Final methanol concentration in the reaction mixture was 1% (v:v). In order to stop the reaction, the same volume of cold acetonitrile containing O-desmethyltramadol-D6 as an internal standard was added. The mix was then centrifuged 3 minutes at 10 000 *g* (Centrifuge 5417C, Eppendorf, Hamburg, Germany), and the supernatant was diluted in water/acetonitrile 80:20 (v:v) for analysis.

2.4 | Reversible inhibition by duloxetine

To assess the reversible inhibition of CYP2D6 by duloxetine, various concentrations of duloxetine (0, 1, 2, 4, 8, 16, 32 and 64 µmol/L) were incubated with dextromethorphan at various concentrations (1, 5, 10, 20 and 50 µmol/L), HLMs (final protein concentration 0.5 mg/mL) and the NADPH generating system described above in phosphate buffer 0.1 mol/L pH 7.4 for 20 minutes. following 3-min.

preincubation time at 37°C. Reactions were then quenched with cold acetonitrile containing O-desmethyltramadol-D6 as an internal standard. Samples were then processed as described above.

2.5 | Time-dependent inhibition (TDI) by paroxetine

A two-step incubation scheme was used in order to investigate the TDI of CYP2D6 by paroxetine. During the preincubation period, mixtures containing paroxetine (0, 0.1, 0.2, 0.5, 1, 2, 5 and 10 µmol/L), HLMs (protein concentration 1.25 mg/mL) and NADPH generating system (described above) in phosphate buffer 0.1 mol/L pH 7.4 were incubated in a 37°C water bath for 0, 4, 8, 12, 16 and 20 minutes. At the appropriate time-points, aliquots of each reaction were transferred (dilution 1:10) into a second reaction mixture containing fresh NADPH generating system and dextromethorphan (final concentration 50 µmol/L) in phosphate buffer 0.1 mol/L pH 7.4. Both 1:10 dilution and saturating concentration of dextromethorphan enabled minimization of CYP2D6 inhibition in the second incubation step. The second incubation was quenched after 10 minutes. at 37°C with cold acetonitrile containing O-desmethyltramadol-D6 as an internal standard and the samples were processed as described above.

2.6 | Liquid chromatography tandem mass spectrometry analysis

Dextrorphan was quantified by high-performance liquid chromatography tandem mass spectrometry (LC-MS/MS). For clearance and duloxetine inhibition experiments, chromatographic separation was performed following 20 µL injection in an HPLC system Agilent series 1100 (Waldbronn, Germany) with a Kinetex™ RP C18 column (Phenomenex, CA, USA) preceded by a Krudkatcher™ ULTRA HPLC In-Line 0.5 µmol/L depth filter (Brechbühler, Echallens, Switzerland). Mobile phases were A) water + formic acid 0.1% + acetonitrile 0.5% (v/v) and B) acetonitrile + formic acid 0.1% (v/v). Initial HPLC condition was composed of 0% of mobile phase B, and then, mobile phase B was increased by a gradient from 0.01 to 2 minutes up to 90%. This condition was kept for 1 minutes. before return to the initial condition until the end of the run (total run time of 7 minutes). Mobile phase flow rate was set at 0.5 mL/min. MS/MS detection was performed on an API 4000 triple quadrupole mass spectrometer controlled by Analyst 1.6.1 software (AB Sciex, Concord, ON, Canada). Mass spectrometry was operated in a multiple reaction monitoring (MRM) mode with electrospray ionization in a positive mode. Transition for dextrorphan and the internal standard O-desmethyltramadol-d6 were 258.2 → 157.0 and 256.0 → 64.0, respectively.

For paroxetine inhibition experiment, the analysis was performed with a different LC-MS/MS method. Following injection of 10 µL, chromatographic separation was performed in an HPLC system Agilent series 1290 (Waldbronn, Germany) with a Kinetex™ RP C18 column (Phenomenex, CA, USA) preceded by a Krudkatcher™ ULTRA HPLC In-Line 0.5 µmol/L depth filter (Brechbühler, Echallens, Switzerland). Mobile phases were A) water + acetic acid 0.1% (v/v) and B) acetonitrile + acetic acid 0.1% (v/v). Initial HPLC condition was composed of 2% of mobile phase B, and then, mobile phase B was increased by a gradient from 1 to 3 minutes up to 90%. This condition was kept for 1 minutes before return to the initial condition until the end of the run (total run time of 5.5 minutes). Mobile phase flow rate was set at 0.6 mL/min MS/MS detection was performed on a QTRAP 6500 triple quadrupole mass spectrometer controlled by Analyst 1.6.1 software (AB Sciex). Mass spectrometry was operated in MRM mode with electrospray ionization in a positive mode. Transitions for dextrorphan and the internal standard O-desmethyltramadol-D6 were the same as described above. The lower limit of quantification (LLOQ) of dextrorphan quantification was 0.1 ng/mL, and calibration curve was linear from 0.1 to 50 ng/mL. For both methods, quality controls' accuracy was within 85% and 115% and intraday and interday variabilities did not exceed 15%.

2.7 | Immunoquantification of CYP2D6 in human liver microsomes

CYP2D6 was quantified in human liver microsomes samples in duplicates by a sandwich ELISA method with a commercially available kit purchased from Biomatik (Wilmington, DE, USA). The provided standard was highly purified *E. coli*-expressed recombinant human CYP2D6. Samples were diluted 400-fold in phosphate buffer 0.1 mol/L pH 7.1 and the concentrations of standards were set between 1.25 and 20 ng/mL. A 3-parameter calibration curve was drawn with CurveExpert 1.4 and used for protein quantification.

2.8 | Data analysis

Kinetic parameters K_M and V_{max} were calculated by nonlinear regression according to the Michaelis-Menten equation (Equation 1), where the initial velocity of the reaction v is a function of the maximal velocity of the substrate (V_{max}), of the concentration of substrate [S], and K_M, the concentration of substrate that reaches half V_{max}.

$$v = \frac{V_{max} \cdot [S]}{K_M + [S]} \quad (1)$$

Intrinsic clearance Cl_{int} was calculated from the ratio of V_{max} over K_M.

Reversible inhibition constant K_i of duloxetine was calculated by non-linear regression using the following equation for competitive inhibition:

$$v = \frac{V_{max} \cdot [S]}{[S] + K_M \left(1 + \frac{[I]}{K_i}\right)} \quad (2)$$

where $[I]$ is the concentration of inhibitor in the reaction mixture.

TDI parameters k_{inact} and K_I were determined using non-linear regression using the following equation:

$$k_{obs} = \frac{k_{inact}[I]}{K_I + [I]} \quad (3)$$

where k_{obs} represents the inactivation rate for a given concentration of the inhibitor in the preincubation mixture and is calculated from the negative slope of the natural logarithm of the percentage of activity remaining after incubation of the inhibitor as a function of preincubation time, k_{inact} is the maximal inactivation rate when $[I]$ reaches infinity and K_I is the inhibitor concentration for which $k_{obs} = 0.5 \cdot k_{inact}$.

All kinetic and inhibition parameters were calculated using GraphPad Prism software version 7 (La Jolla, CA, USA).

2.9 | Statistics

All incubations in the study were performed in duplicates. Results are presented as means ± SD or means with 95% confidence intervals. Kruskal-Wallis H test was used to compare more than 2 independent variables and Mann-Whitney U test was used to compare 2 independent variables. Correlation analysis was performed with a non-parametric Spearman test. A value of 0.05 was set as the significant α level. All statistical analyses were performed with SPSS Statistics, version 24 (Chicago, IL, USA).

2.10 | IVIVE of DDIs

Predicted in vivo magnitudes of DDIs were calculated from Equations (4) and (5) for duloxetine and paroxetine, respectively, according to the US Food and Drug Administration (FDA) guidance on In vitro Metabolism- and Transporter-Mediated Drug-Drug Interaction Studies.[13]

$$\frac{AUC_i}{AUC} = \frac{1}{\left[\frac{1}{1+\frac{[I]_h}{K_i}}\right] \cdot f_{m,CYP2D6} + \left(1 - f_{m,CYP2D6}\right)} \quad (4)$$

$$\frac{AUC_i}{AUC} = \frac{1}{\left[\frac{k_{deg,h}}{k_{deg,h} + \frac{[I]_h \cdot k_{inact}}{[I]_h + K_I}}\right] \cdot f_{m,CYP2D6} + \left(1 - f_{m,CYP2D6}\right)} \quad (5)$$

where AUC_i/AUC represents the ratio of Area Under the Curve (AUC) in the presence of the inhibitor over the AUC in the absence of the inhibitor, $[I]_h$ is the intracellular hepatic concentration of the inhibitor, $f_{m,CYP2D6}$ is the fraction of drug elimination depending on the CYP2D6 pathway, k_{deg} is the apparent first-order degradation rate of enzyme affected by CYP inhibition in the liver.

The intracellular concentrations $[I]_h$ were calculated from maximal blood concentrations $[I]_{max,p}$ taken from[11] as described in Equation 6:

$$[I]_h = f_{u,b} \cdot \left([I]_{max,p} \cdot B/P + F_a \cdot k_a \cdot \frac{Dose}{Q_h}\right) \quad (6)$$

$f_{u,b}$ is the unbound fraction of the drug in blood and was set as 0.05[14] and 0.0893 (predicted from QSAR in Simcyp version 15) for paroxetine and duloxetine, respectively, B/P is the blood plasma ratio and was set to 1.26 and 0.917 for paroxetine and duloxetine respectively (Simcyp version 15), F_a is the fraction absorbed after oral administration, which was set to 0.93746 (Simcyp version 15) and 1 (default value) for paroxetine and duloxetine, respectively, k_a is the first-order absorption rate in vivo, which was set to 0.016978 (Simcyp version 15) and 0.01 min^{-1}[15] for paroxetine and duloxetine, respectively, and Q_h is the liver blood flow, set a 1.45 L min^{-1}.[16]

In order to improve IVIVE, the parameters K_i, K_I and k_{inact} were corrected for microsomal non-specific binding by multiplying the obtained inhibition parameters by the unbound microsomal fraction ($f_{u,mic}$) in the conducted experiments. For paroxetine, the $f_{u,mic}$ was estimated to 0.10 based on previously published microsomal binding experiments.[17] The $f_{u,mic}$ of duloxetine in the competitive inhibition experiments was estimated to 0.47 (calculated with Simcyp f_{umic} calculator, version 16, Certara, NJ, USA). A $f_{m,CYP2D6}$ value of 0.95 for dextromethorphan in CYP2D6 EMs was taken from Simcyp simulator. k_{deg} value of 0.000226 min^{-1} was taken from available in vivo data.[16]

3 | RESULTS

3.1 | Enzyme kinetics

Individual values of Michaelis-Menten kinetic parameters and of intrinsic clearances of dextromethorphan O-demethylation are presented in Table 1. Kinetic parameters calculated in the present study are concordant with a previous study in three extensive metabolizers that estimated K_M between 2 and 9 µmol/L and V_{max} between 70 and 230 pmol min^{-1} mg^{-1} protein.[18] No significant effect of activity score was found for K_M and Cl_{int}. Higher V_{max} values were observed when increasing CYP2D6 activity score, although the single significant difference was the one comparing AS 1 and AS 2 genotypes. Indeed, a 33% smaller maximal reaction velocity was observed in AS 1 than in AS 2 genotypes ($P = 0.006$). Compared to the *1/*1 wild-type genotype, K_M was significantly higher in *2/*2 ($P = 0.009$), *2/*41 ($P = 0.020$) and *2/*3 genotypes ($P = 0.026$). Figure 1 shows Michaelis-Menten kinetics for dextromethorphan O-demethylation in each individual donor.

3.2 | Enzyme Inhibition by duloxetine and paroxetine

Figure 2 shows representative plots for quantitative determination of inhbitory parameters of duloxetine and paroxetine. Duloxetine is known to competitively inhibit CYP2D6 with a reported Ki of 4.5 µmol/L.[19] In the present study, calculated K_i values were close to the one reported previously. The inhibition constants were not significantly different between the three genetic groups. Table 1 presents the K_i values obtained for duloxetine in each individual HLM batch.

Paroxetine is a time-dependent inhibitor of CYP2D6, as indicated by previous experiments showing an IC_{50} (i.e, half maximal inhibitory concentration) shift from 1.5- to 12-fold when paroxetine was pre-incubated for 30 minutes with HLMs as compared to a condition without preincubation (data not shown). Present experiments showed k_{inact} of around 8 h^{-1} and K_I of around 2 µmol/L, which are close to previously reported values.[20] Those inhibitory parameters were not significantly different across the AS tested. TDI parameters of paroxetine for all donors are reported in Table 1.

TABLE 1 Individual data of microsomal CYP2D6 abundance measured by ELISA quantification, reaction velocity of dextromethorphan O-demethylation and inhibition by duloxetine and paroxetine

				Reaction velocity in the absence of inhibitors			Inhibition by duloxetine	Inhibition by paroxetine		
CYP2D6 AS	Sample ID	CYP2D6 genotype	Microsomal CYP2D6 abundance (pmol mg $prot^{-1}$)	K_M (µmol/L)	V_{max} (pmol/min/mg prot)	CL_{int} (µL min^{-1} mg $prot^{-1}$)	K_i (µmol/L)	K_I (µmol/L)	k_{inact} (h^{-1})	k_{inact}/K_I
1	H0428	*1/*4	6.1	5.6 ± 1. 1	124.2 ± 6.3	22.2	5.3 ± 0.5	4.4 ± 0.5	10.3 ± 0.5	2.3
	H0443	*1/*4	8.5	4.0 ± 0.4	76.2 ± 2.1	19.1	4.2 ± 0.4	1.3 ± 0.3	6.6 ± 0.4	5.1
	H0454	*1/*3	6.0	3.7 ± 0.3	98.3 ± 1.9	26.3	2.0 ± 0.3	1.5 ± 0.4	7.1 ± 0.6	4.9
	H0511	*1/*5	10.8	7.6 ± 0.8	107.6 ± 3.0	14.2	2.8 ± 0.3	1.2 ± 0.2	6.8 ± 0.4	5.5
	H0538	*2/*3	10.8	12.5 ± 1.4	69.0 ± 2.5	5.5	7.9 ± 0.7	0.8 ± 0.2	4.2 ± 0.3	5.2
	H0539	*1/*4	10.0	3.5 ± 0.3	130.2 ± 3.1	37.5	2.5 ± 0.3	1.5 ± 0.1	7.7 ± 0.2	5.1
1.5	H0397	*2/*41	15.1	9.1 ± 1.2	147.6 ± 5.7	16.2	4.5 ± 0.6	2.4 ± 0.7	7.0 ± 0.7	2.9
	H0464	*1/*41	8.0	5.9 ± 0.6	107.1 ± 2.8	17.9	1.9 ± 0.3	1.8 ± 0.3	9.1 ± 0.5	5.0
	H0500	*2/*41	10.0	7.9 ± 1.0	75.9 ± 2.6	9.6	5.6 ± 0.4	2.4 ± 0.7	8.2 ± 0.9	3.5
	H0535	*1/*41	11.2	6.7 ± 0.7	134.5 ± 3.8	20.1	5.5 ± 0.6	3.2 ± 1.0	6.8 ± 0.9	2.1
2	H0447	*1/*1	5.8	4.1 ± 0.5	126.8 ± 4.0	30.9	4.1 ± 0.4	2.9 ± 1.1	9.8 ± 1.4	3.4
	H0485	*1/*2	6.9	8.1 ± 0.8	169.0 ± 5.0	20.9	1.5 ± 0.2	1.4 ± 0.4	8.2 ± 0.6	5.7
	H0493	*1/*1	8.3	2.7 ± 0.4	157.8 ± 5.8	59.2	3.7 ± 0.4	1.5 ± 0.4	6.7 ± 0.5	4.4
	H0509	*2/*2	12.2	11.3 ± 1.1	141.1 ± 4.1	12.5	4.9 ± 0.4	0.9 ± 0.2	6.1 ± 0.5	6.9
	H0512	*2/*2	6.0	14.4 ± 1.1	155.5 ± 4.0	10.8	4.3 ± 0.4	1.6 ± 0.4	6.8 ± 0.6	4.2
	H0520	*1/*1	8.7	5.1 ± 0.9	160.0 ± 7.5	31.6	2.2 ± 0.3	6.6 ± 1.5	11.0 ± 1.3	1.67

Results for K_M, V_{max}, K_i, K_I and k_{inact} are presented as means ± SD, as output from GraphPad Prism analysis.

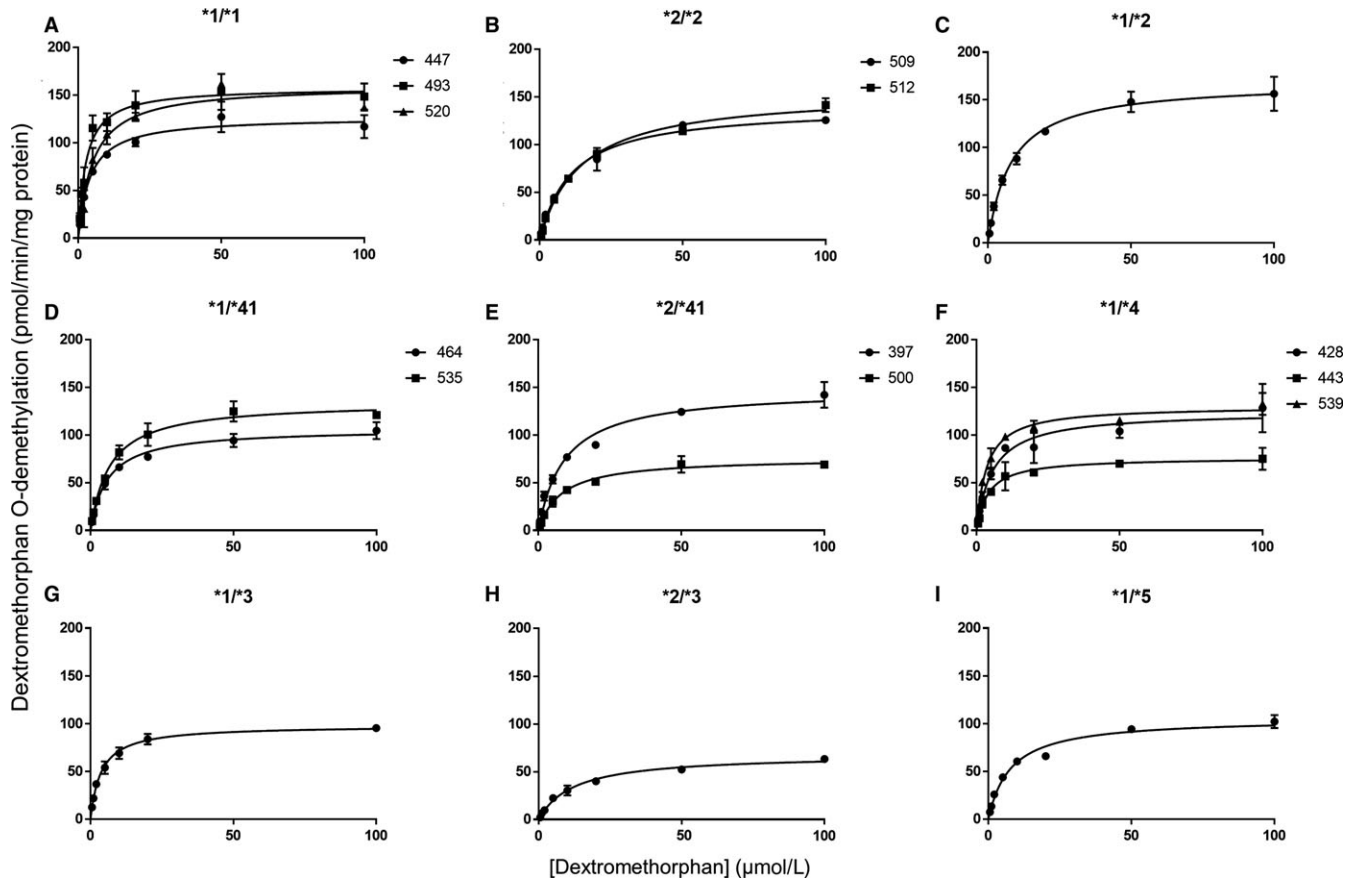

FIGURE 1 Dextromethorphan O-demethylation kinetics in various CYP2D6 genotypes tested. *1/*1 (n = 3, A), *1/*2 (n = 1, C) and *2/*2 (n = 2, B) constitute the AS 2 genetic group, *1/*41 (n = 2, D) and *2/*41 (n = 2, E), the AS 1.5 genetic group, and *1/*4 (n = 3, F), *1/*3 (n = 1, G), *2/*3 (n = 1, H) and *1/*5 (n = 1, I) the AS 1 genetic group. Error bars show standard deviation of duplicate observations

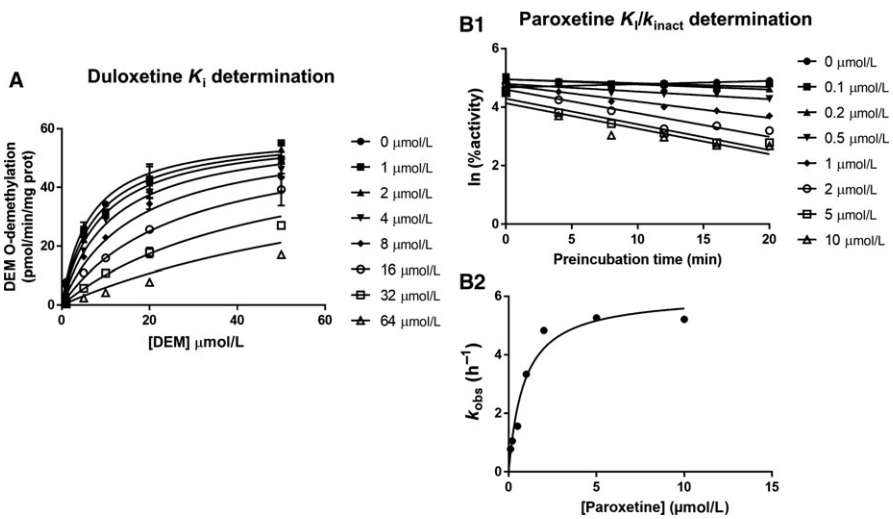

FIGURE 2 Representative plots (sample H0509) for (A) duloxetine K_i (dextromethorphan (DEM) O-demethylation as a function of dextromethorphan concentration for multiple duloxetine concentrations) and (B) paroxetine K_I and kinact determinations (b1—natural logarithm of percentage activity remaining as a function of preincubation time for multiple paroxetine concentrations and b2 —k_{obs} values plotted as a function of paroxetine concentrations)

A summary of mean kinetic and inhibitory parameters assessed in the present study is given in Table 2. Figure 3 shows a comparison of kinetic parameters K_M, V_{max} and Cl_{int}, and Figure 4 inhibition parameters of duloxetine and paroxetine for each genotype tested.

3.3 | CYP2D6 microsomal quantification

Immunoquantification of CYP2D6 using a sandwich ELISA method showed a mean abundance of 9.0 pmol/mg total protein in liver microsomes (CV 29%), which is concordant with previously published data.[21] There was no

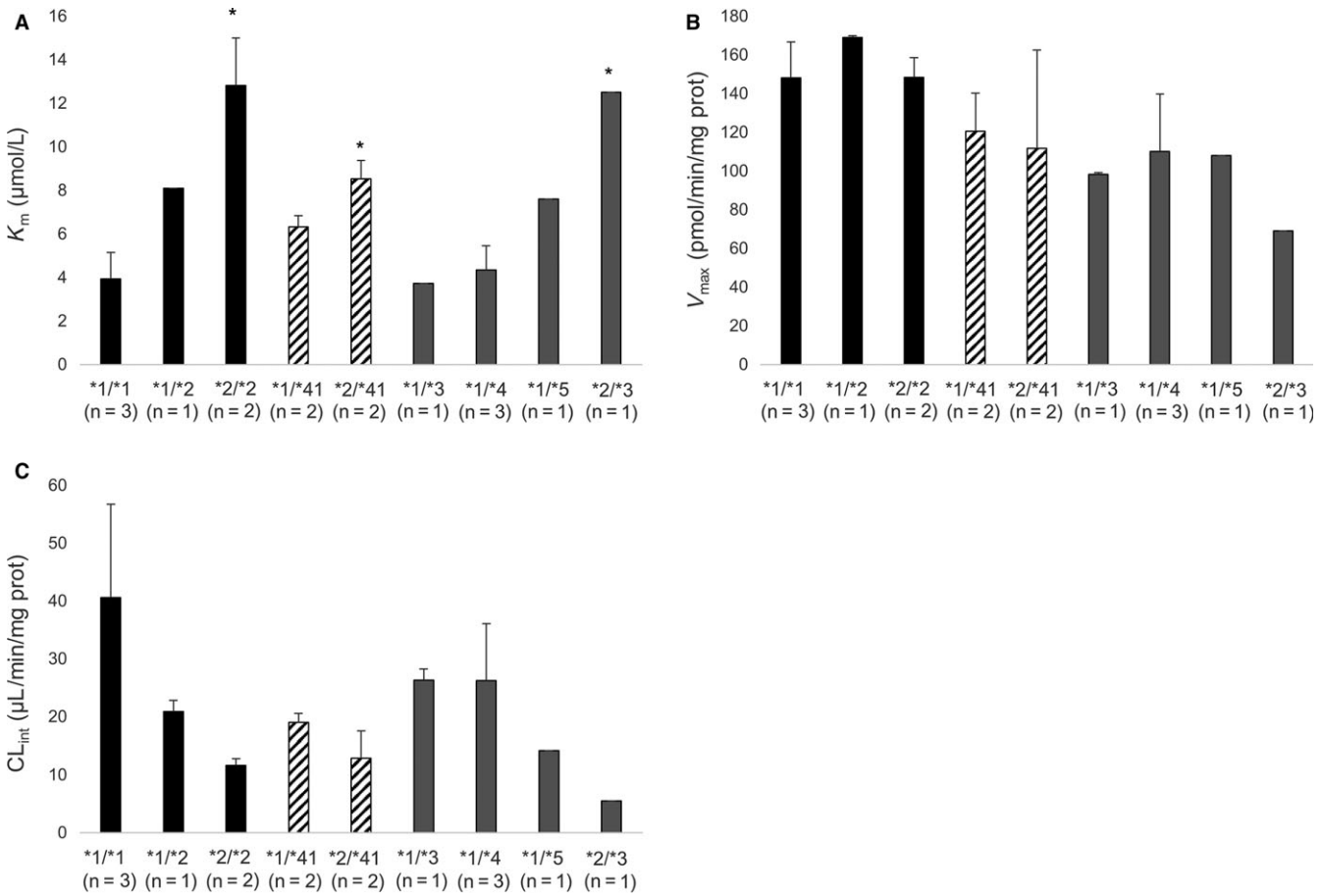

FIGURE 3 Comparison of dextromethorphan O-demethylation kinetic parameters K_M (A), V_{max} (B) and Cl_{int} (C) among the tested genotypes. Black boxes indicate AS 2 genotypes, hatched boxes indicate AS 1.5 genotypes and grey boxes indicate AS 1 genotypes. * indicates that a significant difference ($P < 0.05$) was observed compared to the *1/*1 genotype

effect of genotype on CYP2D6 levels ($P = 0.257$). Likewise, no correlation was found between CYP2D6 activity (Cl_{int}) and CYP2D6 microsomal abundance ($\rho = -0.389$, $P = 0.136$).

3.4 | IVIVE of DDIs

For the effect of duloxetine on the AUC of dextromethorphan, AUC ratios of 1.07 and 1.09 were predicted based on our in vitro results, while AUC ratios of 1.8 ($CI_{95\%}$: 1.5-2.1) and 2.4 ($CI_{95\%}$: 1.8-3.2) were observed in vivo in AS 1 and AS 2 healthy volunteers, respectively.[11]

With paroxetine, AUC ratios of dextromethorphan were estimated between 16.1 and 16.6. In vivo, values of 8.5 ($CI_{95\%}$: 6.7-10.8) and 14.6 ($CI_{95\%}$: 10.0-21.4) were observed in AS 1 and AS 2 healthy volunteers, respectively. When $f_{m,CYP2D6}$ was isolated from Equation (4), the values that best fitted to the observed AUC ratios considering the inhibition parameters estimated in the present study were 0.89 ($CI_{95\%}$: 0.86-0.92) and 0.94 ($CI_{95\%}$: 0.91-0.96) for AS 1 and AS 2, respectively.

4 | DISCUSSION

Previously, Shimada and colleagues reported high interindividual variations in HLMs from Japanese, Chinese and Caucasians individuals, even across a same genotype.[22,23] The highlighted intra-genotype variability suggests the implication of non-genetic factors affecting CYP2D6 activity. Yet some genetic variants have shown their ability to affect the intrinsic clearance of CYP2D6 substrates. The *10/*10 genotypes in Japanese individuals, characterized by a homozygous 100 C>T mutation, were distinguished by a reduced (>6-fold) intrinsic clearance compared to the wild-type genotype.[22] This was further confirmed by another study that demonstrated decreased enzyme affinity (>2-fold increase in K_M) and intrinsic clearance (2.9-fold) in HLMs from subjects carrying the 100TT SNP compared to wild-type.[24] Previously, it was reported that one homozygous carrier of the *CYP2D6**4 allele had markedly reduced intrinsic clearance compared to wild-type subjects.[25] The present study did not highlight any difference in intrinsic clearances and enzyme affinity (K_M) in the

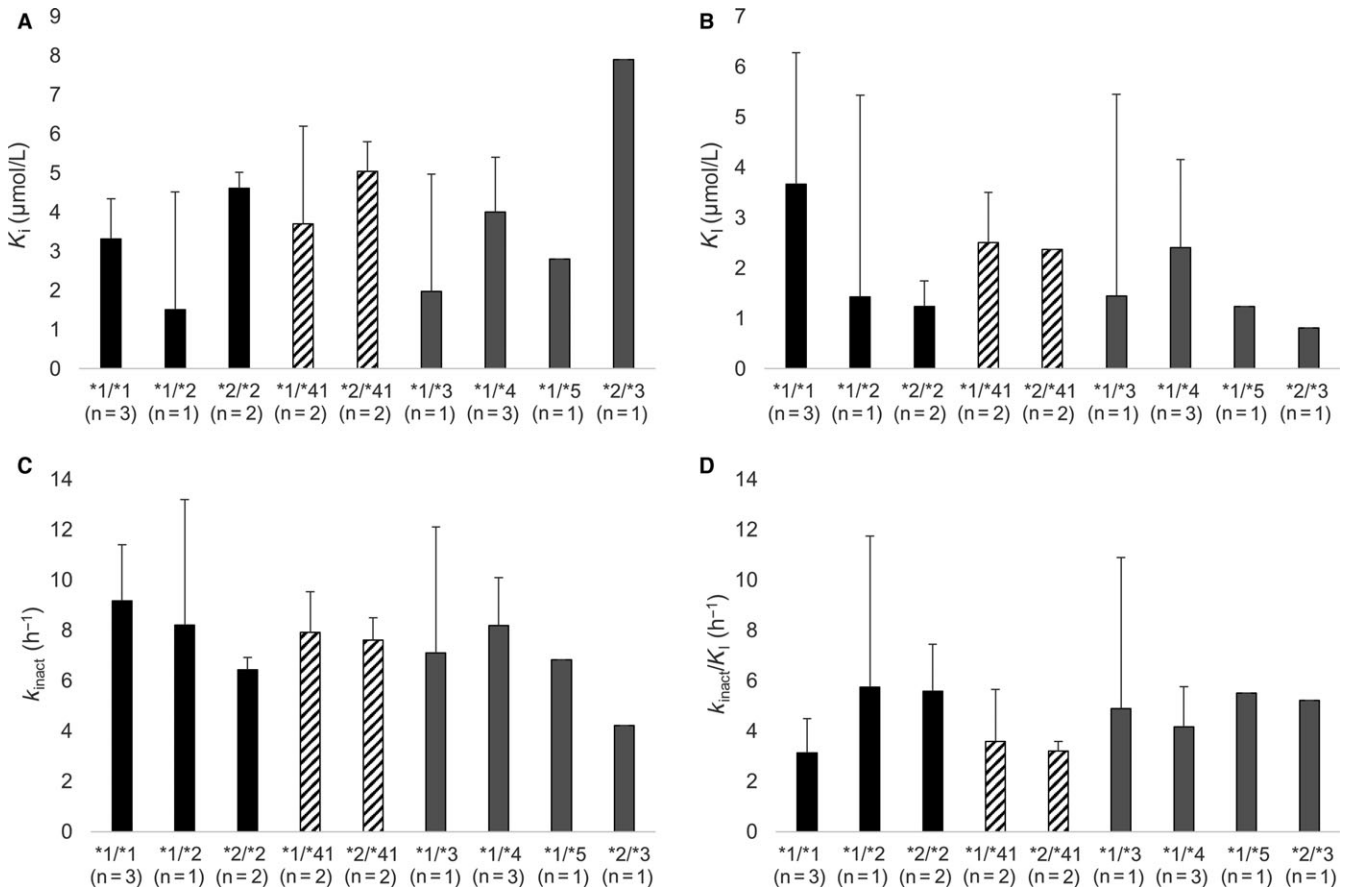

FIGURE 4 Comparison of competitive (duloxetine, A) and time-dependent (paroxetine, B-D) inhibition parameters towards dextromethorphan O-demethylation among the tested genotypes. Black boxes indicate AS 2 genotypes, hatched boxes indicate AS 1.5 genotypes and grey boxes indicate AS 1 genotypes

various genotypes tested, which all predict extensive metabolizer phenotypes according to the CPIC classification. An effect of genotype on maximal reaction velocity was observed, with maximal velocity being decreased by 23% in AS 1.5 and 33% in and AS 1 compared to AS 2 subjects (Table 2). This difference in reaction velocities could not be related to CYP2D6 protein abundance, as measured by ELISA immunoquantification. There are indeed multiple limitations of immunoquantification methods, including the inability of the antibody to differentiate between the wild-type enzyme and its genetic variant and sensibility of the quantification results towards standard sources, which have a considerable impact on holoprotein/total protein ratio.[26] Indeed, only holoprotein (active form of the enzyme as conjugate with the prosthetic group) is relevant for the IVIVE of drug clearance and DDIs, whereas total protein (active and inactive) are measured by immunoquantification methods, which might explain the lack of correlation between protein abundance estimates and CYP2D6 activity that was observed in the present study. This is of particular interest in cases where genetic variants are present, as it was shown that genotype has an impact on the holoprotein/total protein ratio.[27]

A few in vitro studies have previously highlighted differential CYP inhibition as a function of genotype. Those mostly concern CYP2B6 and CYP2C9, even though some data exist for CYP2D6. *CYP2B6* variant alleles *4 (leading to increased function) and *6 (leading to decreased function) have also been studied in regard to CYP inhibition. The results are discordant, which seems related to the substrate-inhibitor couples used in the experiments. While decreased inhibition of bupropion metabolism by 17α-ethinylestradiol and efavirenz was observed in homozygous carriers of the *CYP2B6**6 variant allele,[28] higher inhibition potency of efavirenz metabolism by voriconazole was demonstrated in HLMs with the *6 allele.[29] To note, a minor fraction of efavirenz is metabolized by CYP3A,[30] which can also be inhibited by voriconazole.[31] The *6 allele is characterized by a non-synonymous SNP leading to the substitution of a lysine by an arginine in position 262 (K262R), which is also found in the *4 allele, and by another non-synonymous SNP leading to the substitution

TABLE 2 Summary of velocity and inhibitory parameters

	CYP2D6 activity score			
	1 (n = 6)	**1.5 (n = 4)**	**2 (n = 6)**	***P*-value[a]**
Dextromethorphan O-demethylation reaction velocity				
K_M (μmol/L)	6.1 (2.5-9.8)	7.4 (5.2-9.7)	7.6 (2.8-12.4)	0.511
V_{max} (pmol min^{-1} mg^{-1} $prot^{-1}$)	101 (75-127)	116 (66-167)	152 (136-168)[a]	0.014
Cl_{int} (μL min^{-1} mg^{-1} $prot^{-1}$)	20.8 (9.4-32.2)	15.9 (8.7-23.2)	27.7 (9.0-46.3)	0.426
Competitive inhibitory parameter of duloxetine				
K_i (μmol/L)	4.1 (1.8-6.4)	4.4 (1.7-7.1)	3.4 (2.1-4.8)	0.520
Time-dependent inhibitory parameters of paroxetine				
K_I (μmol/L)	1.8 (0.4-3.2)	2.4 (1.5-3.4)	2.5 (0.3-4.7)	0.189
k_{inact} (h^{-1})	7.1 (5.1-9.2)	7.8 (6.0-9.5)	8.1 (6.0-10.1)	0.728
k_{inact}/K_I (h^{-1} μM^{-1})	4.7 (3.5-5.9)	3.4 (1.4-5.4)	4.4 (2.5-6.3)	0.288

All values are expressed as arithmetic means with 95% confidence intervals.
[a]Kruskal-Wallis *H* test.

of a glutamine by a histidine in position 172 (Q172H). The K262R variant leads to increased substrate turnover, while Q172H decreases enzyme expression in relation to alternative mRNA splicing.[32] Talakad and colleagues highlighted a lower susceptibility to 7-MFC O-demethylation inhibition by sertraline and clopidogrel with the K262R isolated variant and the Q172H/K262R double variant compared to wild-type enzyme.[33] Likewise, *CYP2C9* variant alleles *3 and *13 have been associated with decreased inhibition potencies of fluvastatin and fluvoxamine towards diclofenac 4′-hydroxylation compared to wild-type enzyme.[34] It had previously been demonstrated that the inhibition potency of a wide range of CYP2C9 inhibitors was dependent on both genotype and substrate-inhibitor couples, therefore suggesting multiple binding regions within the enzyme active site.[35] Regarding CYP2D6, the strong inhibitor quinidine showed lower inhibition potency of bufuralol 1′-hydroxylation in homozygous carriers of the *CYP2D6**10 allele compared to homozygous carriers of functional *1 and *2 alleles, and did not show any inhibition potency in homozygous carriers of the *4 allele,[22] which can be explained by the fact that the *4 allele leads to non-functional enzyme variant, which therefore cannot be inhibited any further. In the present study, we tested different genotypes leading to activity scores of 1 to 2 towards competitive and time-dependent CYP2D6 inhibition. We observed important intra-genotype variability (Table 1) and could not highlight any difference in the inhibitory potencies of duloxetine and paroxetine (Table 2). While we recognize that the small sample size of the present in vitro study may represent a limitation to draw firm conclusions, our data do not seem to show any tendency towards any significant effect of genotype on inhibitory parameters among the three AS tested. It should, however, be mentioned that if not AS-specific, the effect of inhibitors might rather be allele specific, which could be tested using recombinant enzymes as previously reported for CYP2C9 substrates and inhibitors.[36] As an example, the CYP2D6*17 allele has shown some substrate-specific effects, which is probably explained by a modification of ligand-binding properties in the variant enzyme. This indicates that inhibitor-specific effects cannot be ruled out for this allele, which unfortunately was not tested in the present research.

In the in vivo study that motivated the present in vitro investigations, we previously showed differential magnitudes of interaction as a function of genotype. Indeed with the moderate inhibitor duloxetine, a lower, although no statistically significant, ratio of dextromethorphan AUC was observed in AS 1 as compared to AS 2 healthy volunteers (1.8 vs 2.4, $P > 0.05$). With the strong inhibitor paroxetine, the AUC ratio was decreased by >40% in AS 1 subjects compared to AS 2 (14.6 vs 8.5, $P < 0.05$).[11] The results of the present study suggest that this difference in magnitude of DDI cannot be explained by differential inhibitory potencies at the microsomal level but more probably by the amount of functional protein in the liver. This parameter has an impact on the contribution of the enzyme in total drug clearance, which is highly important for the IVIVE of DDIs, as seen in Equations 4 and 5 described above. This is supported by the present observation of an effect of genotype on maximal reaction velocity. Indeed, the higher the maximal velocity the faster will be the enzymatic capacity and the more the substrate clearance will depend on the enzyme. The explanation for such difference in maximal reaction velocities might be attributed to

functional CYP2D6 abundance. However, as discussed above, we could not identify any difference in microsomal CYP2D6 abundance. While mass spectrometry suffers from the same limitations regarding the holoprotein content, it still provides a more precise quantification and might allow differential quantification of wild-type and genetic variant of CYP2D6, thus representing a promising perspective to improve the IVIVE of gene-drug-drug interactions.

Regarding the IVIVE of dextromethorphan-duloxetine interaction, we observed an underprediction of the AUC ratio. Explanations might involve an accumulation process of duloxetine into hepatocytes, which was not taken into account in the IVIVE. Unfortunately, no data concerning any cellular accumulation of duloxetine could be found in the available literature. In addition, the $f_{u,mic}$ value of duloxetine in inhibition experiments was estimated based on the molecule's physicochemical properties and was not determined experimentally. There is clear evidence that the prediction of DDI can be significantly improved by accounting for intracellular unbound drug concentration, which can be estimated using isolated perfused livers or sandwich-cultured hepatocytes.[37]

In conclusion, the present study results suggest that the corresponding different values of f_m calculated from the observed AUC ratios for the paroxetine-dextromethorphan DDI are likely to be due to differential amounts of functional proteins at the microsomal level. These conclusions motivate for further proteomic quantification of functional and variant CYP2D6 enzymes in HLMs to improve the prediction of genotype-dependent DDIs.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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