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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 $V_{\text{max}}$ in AS 1 HLMs compared to AS 2 ($P = 0.06$). No difference in inhibition parameters $K_i$, $K_I$ and $k_{\text{inact}}$ 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 immunquantification. 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. A huge variability in the activity of CYP2D6 has been observed in the general population and is mainly explained by genetic polymorphisms and DDIs. More than one hundred alleles have been identified and described so far. 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.

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. Indeed, several in vivo studies showed differential magnitudes of DDIs in EM, IM and UM subjects. 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.

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 (MgCl2), isocitratereductohydrogenase 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, 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°C-warmed water bath, the reaction was initiated by addition of a NADPH generating system (NADP 4 mmol/L, isocitrate 15 mmol/L, MgCl2 12.5 mmol/L and isocitratereductohydrogenase 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-desmethyltrama-dol-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

Dextrophan 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 + 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 dextrophan and the internal standard O-desmethyltramadol-d6 were the same as described above. The lower limit of quantification (LLOQ) of dextrophan 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 $V_{max}$. 

$$ v = \frac{V_{max} [S]}{K_M + [S]} $$
\[ V = \frac{V_{\text{max}}[S]}{K_M + [S]} \]  \hspace{1cm} (1)

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

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

\[ V = \frac{V_{\text{max}}[S]}{[S] + K_M \left( 1 + \frac{[I]}{K_i} \right)} \]  \hspace{1cm} (2)

where \([I]\) is the concentration of inhibitor in the reaction mixture.

TDI parameters \( k_{\text{inact}} \) and \( K_i \) were determined using non-linear regression using the following equation:

\[ k_{\text{obs}} = \frac{k_{\text{inact}}[I]}{K_i + [I]} \]  \hspace{1cm} (3)

where \( k_{\text{obs}} \) 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, \( k_{\text{inact}} \) is the maximal inactivation rate when \([I]\) reaches infinity and \( K_i \) is the inhibitor concentration for which \( k_{\text{obs}} = 0.5 \cdot k_{\text{inact}} \).

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 \( \alpha \) 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.\(^\text{13}\)

\[
\frac{\text{AUC}_i}{\text{AUC}_i} = \frac{1}{\frac{1}{f_{m,CYP2D6}} + \left( 1 - f_{m,CYP2D6} \right)}
\]  \hspace{1cm} (4)

\[
\frac{\text{AUC}_i}{\text{AUC}_i} = \frac{1}{\frac{k_{\text{deg,h}}}{k_{\text{deg,h}}^{\text{max}} + \left( \frac{f_{m,CYP2D6}}{f_m,CYP2D6} \right)}^2}
\]  \hspace{1cm} (5)

where \( \text{AUC}_i/\text{AUC} \) 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, \( k_{\text{deg}} \) 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 \([I]_{\text{max,p}}\) taken from\(^\text{11}\) as described in Equation 6:

\[
[I]_h = \frac{f_{u,b}}{f_{u,m}} \frac{B/P}{f_a} \left( \frac{K_i}{C_1^{\text{mic}}} \frac{\text{Dose}}{Q_h} \right)
\]  \hspace{1cm} (6)

\( f_{u,b} \) is the unbound fraction of the drug in blood and was set as \( 0.05^{\text{14}} \) 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, \( Q_h \) is the liver blood flow, set to \( 1.45 \) L min\(^{-1}\).\(^\text{16}\)

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

<table>
<thead>
<tr>
<th>CYP2D6 AS</th>
<th>Sample ID</th>
<th>CYP2D6 genotype</th>
<th>Microsomal CYP2D6 abundance (pmol mg prot$^{-1}$)</th>
<th>Reaction velocity in the absence of inhibitors</th>
<th>Inhibition by duloxetine</th>
<th>Inhibition by paroxetine</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>$K_M$ (μmol/L)</td>
<td>$V_{max}$ (pmol/min/mg prot)</td>
<td>$Cl_{int}$ (μL min$^{-1}$ mg prot$^{-1}$)</td>
<td>$K_i$ (μmol/L)</td>
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<tr>
<td>1</td>
<td>H0428</td>
<td>*1/*4</td>
<td>6.1</td>
<td>5.6 ± 1.1</td>
<td>124.2 ± 6.3</td>
<td>22.2</td>
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<td>H0443</td>
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<td>8.5</td>
<td>4.0 ± 0.4</td>
<td>76.2 ± 2.1</td>
<td>19.1</td>
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<td>98.3 ± 1.9</td>
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<td>5.1 ± 0.9</td>
<td>160.0 ± 7.5</td>
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</tbody>
</table>

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

Immuoquantification 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. There was no
effect of genotype on CYP2D6 levels ($P = 0.257$). Likewise, no correlation was found between CYP2D6 activity ($Cl_{\text{int}}$) 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 $f_{\text{m,CYP2D6}}$ 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.

### 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
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 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

![FIGURE 4](image-url)
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 that the Q172H/K262R double variant leads to increased substrate turnover, while the AUC ratio was decreased by >40% in AS 1 subjects 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, \( P < 0.05 \)). 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 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, \( P > 0.05 \)). 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 indicates that inhibitor-specific effects cannot be ruled out for this allele, which unfortunately was not tested in the present research.
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 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.\textsuperscript{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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