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Review

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Pharmacogenomics

Substrate specificity of CYP2D6 genetic variants

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Genetic variation in the gene encoding CYP2D6 is used to guide drug prescribing in clinical practice. However, genetic variants in CYP2D6 show substrate-specific effects that are currently not accounted for. With a systematic literature, we retrieved 22 original studies describing *in vitro* experiments focusing on *CYP2D6* alleles (*CYP2D6**1, *2, *10 and *17) and substrates. Allele activity (clearance of the allele of interest divided by the clearance of the wildtype) was extracted. The results support the hypothesis of the existence of substrate specificity of the *CYP2D6**17-allele (higher debrisoquine clearance), a subtle effect of the *CYP2D6**10-allele (lower dextromethorphan clearance) but no substrate-specific effect of the *CYP2D6**2-allele. Although our results support substrate specificity, for most substrates data are too sparse and require further studies.

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Keywords: bufuralol • CYP2D6 • debrisoquine • dextromethorphan • *in vitro* • personalized medicine • substrate specificity • venlafaxine

The CYP2D6 enzyme is involved in the metabolism of 20–30% of commonly prescribed drugs, making it one of the most important enzymes in drug metabolism [1]. The gene encoding the CYP2D6 enzyme, *CYP2D6*, is highly polymorphic and harbors single nucleotide variants as well as structural variants. These variants lead to either increased or decreased enzyme activity [2,3]. To allow for clinical interpretation, variants within the *CYP2D6* gene are translated into haplotypes using the star (*)- nomenclature [3]. Currently, more than 140 *CYP2D6* haplotypes have been described and are cataloged by the Pharmacogene Variation Consortium (PharmVar). Each allele is assigned an allele activity score, with a score of 0 for nonfunctional alleles, 0.25 or 0.5 for decreased activity alleles, 1.0 for fully active alleles and 2.0 for duplications or increased activity alleles. The scores of the paternal and maternal alleles are combined into a gene activity score (GAS) for the subject, which in turn is translated into predicted drug metabolizer phenotypes based on the guidelines from Clinical Pharmacogenetics Implementation Consortium or the Dutch Pharmacogenetics Working Group [4–6].

The activity scores and the genotype-predicted drug metabolizer phenotypes are based on *in vitro* and *in vivo* data. Results from *in vitro* experiments are used to infer activity scores for *CYP2D6* alleles for which data from clinical studies are lacking [3,7,8]. Gene–drug interactions are often supported by high levels of evidence before they are included in the guidelines [9,10]. However, with estimations showing that more than 95% of the variants in pharmacogenes are rare variants [11,12], it is impossible to obtain such high-level evidence for the effect of every single haplotype in the *CYP2D6* gene. Therefore, *in vitro* experiments can be used to assess the activity of (novel) haplotypes and compare this to haplotypes of which the effect is known [7,13]. Even though the Pharmacogenomics Knowledge base (PharmGKB) classifies *in vitro* experiments among the lowest level of evidence, comparable to case reports [13], *in vitro* data can be valuable in gaining insight into the effect of (novel) variants and haplotypes. These results can be used in the greater scope of gene–drug interactions.

Generally, the genotype to phenotype translations from a particular substrate is extrapolated to all CYP2D6 substrates, assuming generalizability for allele activity and for the clinical impact of the phenotype categories [4,5]. For example, if a patient carries a *CYP2D6* *1/*4 genotype, by convention the predicted CYP2D6 phenotype is an intermediate metabolizer regardless of the CYP2D6 substrate [4,5]. However, the extent to which the metabolism

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is affected by a specific genetic variant may differ per substrate. This inter substrate variability is also known as substrate specificity [14–16]. Indeed, pharmacokinetic data show that for tricyclic antidepressants the *CYP2D6**1/*4 diplotype leads to significantly decreased metabolism whereas the metabolism of the CYP2D6 substrate codeine is much less affected [9,17].

In vitro data could help to better characterize and understand the impact of specific variants in CYP2D6 substrate specificity. However, it is well recognized that the use of data from individual *in vitro* studies to predict *in vivo* variant contributions and substrate-specific effects is limited due to the substantial impact of experimental conditions such as the applied vector and incubation concentrations on the results of the *in vitro* experiments [16,18–20]. This restricts the value of individual *in vitro* studies regarding substrate specificity as it cannot be determined if the observed effect is the result of substrate specificity or the experimental design. In this study, we collect data from multiple *in vitro* kinetic studies to assess the impact of *CYP2D6* variants on different substrates with the aim to estimate the role of substrate specificity. Comparisons will be made within one study and experimental system, to correct for study-specific effects.

Methods

Literature search

Studies were selected through a systematic literature search focusing on the CYP2D6 substrates included in the Flockhart table [21]. PubMed was searched to identify and extract relevant papers until December 2020. Search terms consisted of 'CYP2D6', 'functional characterization' and the substrate of interest. Reference lists from reviews were manually checked to identify relevant crossreferences. The full search string is provided in Supplementary File 1. Only papers with full text available in the English language were included. Furthermore, the studies had to include kinetic parameters including the Km (Michaelis constant) and V_{max} (maximum conversion speed).

The study was limited to *CYP2D6* alleles which are included in the *-nomenclature, meaning that no individual (rare) variants were included.

Data extraction

From the selected studies, the following data were extracted: kinetic parameters, incubation specifics and the transfection vector used. Four kinetic parameters were extracted or calculated based on available data: Km, V_{max}, intrinsic clearance (Clint expressed as V_{max}/Km), calculated enzyme activity. The calculated activity was defined as the Clint of the allele of interest normalized to the Clint of the wildtype *1 allele in the same experiment. Individual kinetic parameters (Km, V_{max} and Clint) were highly variable between studies due to study specific effects, resulting in an inability to compare these parameters between studies. To assess the substrate-specific effects per allele, the calculated activity of all substrates was compared. For substrates which were studied in more than two studies, a comparison of multiple drugs within one study was made to assess the relative activity of an allele for the different drugs. This cut-off was made to ensure that the same substrate comparisons could be made in different studies. Moreover, this comparison negated some of the study-specific effects, as these were assumed to be identical within one study. Experiment specific parameters were extracted to determine if these factors result in differences in observed enzyme activity.

All analyses were performed with R, v3.6.3.

Results

The literature search identified 286 papers. After analysis of the full text, 22 studies were found to meet all inclusion criteria (Supplementary Figure 1 & Supplementary Table 1). Manual checking of references identified bufuralol as a commonly used CYP2D6 substrate for *in vitro* experiments and this was added to the substrate selection. The alleles selected were *1, *2, *10 and *17; the impact of these variants on the substrate-binding site is summarized in Supplementary Figure 2, based on findings by Dong *et al.* [22]. Substrates occurring in three or more studies were bufuralol (7 studies), dextromethorphan (8 studies), debrisoquine (5 studies) and venlafaxine (3 studies), hereafter called the common substrates. For another 13 substrates, less than 3 studies were identified (Supplementary Figure 1 & Supplementary Table 2).

Study-specific parameters were collected and assessed on their influence on the observed activity. Investigated parameters were: vector (e.g., baculovirus, yeast cells, microsomes), incubation time and the minimum and maximum substrate concentrations. The majority of these factors did not seem to influence the outcomes in a specific direction (Supplementary Figure 3). The only exception was the use of yeast cells which seems to result in a slightly

Table 1. Relative activity of alleles for common substrates.

		Dextromethorphan	Bufuralol	Debrisoquine	Venlafaxine
*2	Observations (n)	5	5	3	2
	Mean (SD)	0.57 (0.34)	0.77 (0.28)	0.98 (0.17)	0.67 (0.57)
	Median (range)	0.44 (0.19–1.1)	0.74 (0.40–1.18)	1.01 (0.8–1.13)	0.67 (0.26–1.07)
*10	Observations (n)	6	5	2	3
	Mean (SD)	0.037 (0.02)	0.32 (0.38)	0.26 (0.20)	0.24 (0.22)
	Median (range)	0.04 (0.01–0.06)	0.24 (0.01–0.96)	0.26 (0.12–0.4)	0.22 (0.03–0.47)
*17	Observations (n)	5	4	3	1
	Mean (SD)	0.17 (0.058)	0.27 (0.07)	0.52 (0.26)	0.097 (NA)
	Median (range)	0.17 (0.10–0.25)	0.24 (0.21–0.38)	0.64 (0.22–0.7)	0.097 (NA)

Activity is compared with *CYP2D6**1 by dividing the intrinsic clearance of the allele of interest by the intrinsic clearance of the *1 allele within the same experiment. Data are obtained from kinetic parameters of selected *in vitro* studies focusing on dextromethorphan, bufuralol, debrisoquine and venlafaxine.
NA: Not available; SD: Standard deviation.

higher activity of CYP2D6*10 and *CYP2D6**17 compared with the other vectors; however, for *CYP2D6**2, this effect was not observed.

CYP2D6*2

The mean *in vitro* activity of *CYP2D6**2 was 0.65 ± 0.34 (range: 0.17–1.18; Supplementary Table 2). Although this is higher than the activity of *10 and *17, there is also a suggestion of a decrease of activity compared with the wildtype allele. In clinical practice, it is currently assumed that the *2 allele has the same activity as the wildtype allele.

Of the four studies comparing bufuralol and dextromethorphan, two reported a comparable activity of *2 for both substrates [23,24], whereas one reported a decreased activity of the *2 allele for dextromethorphan (Figure 2 & Supplementary Table 1) [25]. Of the two studies focusing on debrisoquine, Marcucci *et al.* reported a decreased activity compared with bufuralol (1.18 compared with 1.01) [24], whereas Bapiro *et al.* reported a slight increase (0.80 compared with 0.74) [25].

For the other substrates, a similar amount of variability is observed, with activities ranging from clear decreased activity for amitriptyline to normal activity for metoprolol (Figure 1). Moreover, for many of the substrates (7/15 substrates), *CYP2D6**2 was a decreased activity allele with an activity around 0.30–0.70, while the activity was comparable to *1 (0.80–1.2) for only 4/15 substrates.

CYP2D6*10

The mean *in vitro* activity of *CYP2D6**10 was 0.21 ± 0.27 (range: 0.01–0.96), which is in line with the current GAS of 0.25. However, a larger amount of variability remains with some studies reporting almost no activity and others normal activity (Supplementary Tables 1 & 2).

For the common substrates, the results are suggestive of a lower *CYP2D6**10 activity in the metabolism of dextromethorphan compared with the other substrates (Table 1 & Figure 1). Nonetheless, by comparing the metabolic activities of the different substrates within studies, this effect is no longer evident. Three studies comparing dextromethorphan to bufuralol report conflicting results (Figure 2 & Supplementary Table 1). Nakamura *et al.* report a lower metabolic activity of *CYP2D6**10 for dextromethorphan compared with bufuralol (0.06 for dextromethorphan and 0.33 for bufuralol) [26]. By contrast, Cai *et al.* and Shen *et al.* report a small increase in metabolic activity for dextromethorphan compared with bufuralol (0.05 and 0.05 for dextromethorphan compared with 0.01 and 0.04 for bufuralol) [23,27].

For the other substrates, a wide range of *CYP2D6**10 activity was observed. For the substrates amitriptyline, codeine, fluoxetine, metoprolol, nortriptyline, propranolol and tramadol the *CYP2D6**10 allele showed an activity below 0.13. In contrast, *CYP2D6**10 activity in regards to mexiletine metabolism (0.78 and 0.93) reflected almost normal function.

CYP2D6*17

On average, the *in vitro CYP2D6**17 activity was 0.28 ± 0.21 (range: 0.06–0.80), which is comparable to the *CYP2D6**10 activity and lower than the currently assigned GAS of 0.5 (Supplementary Table 2).

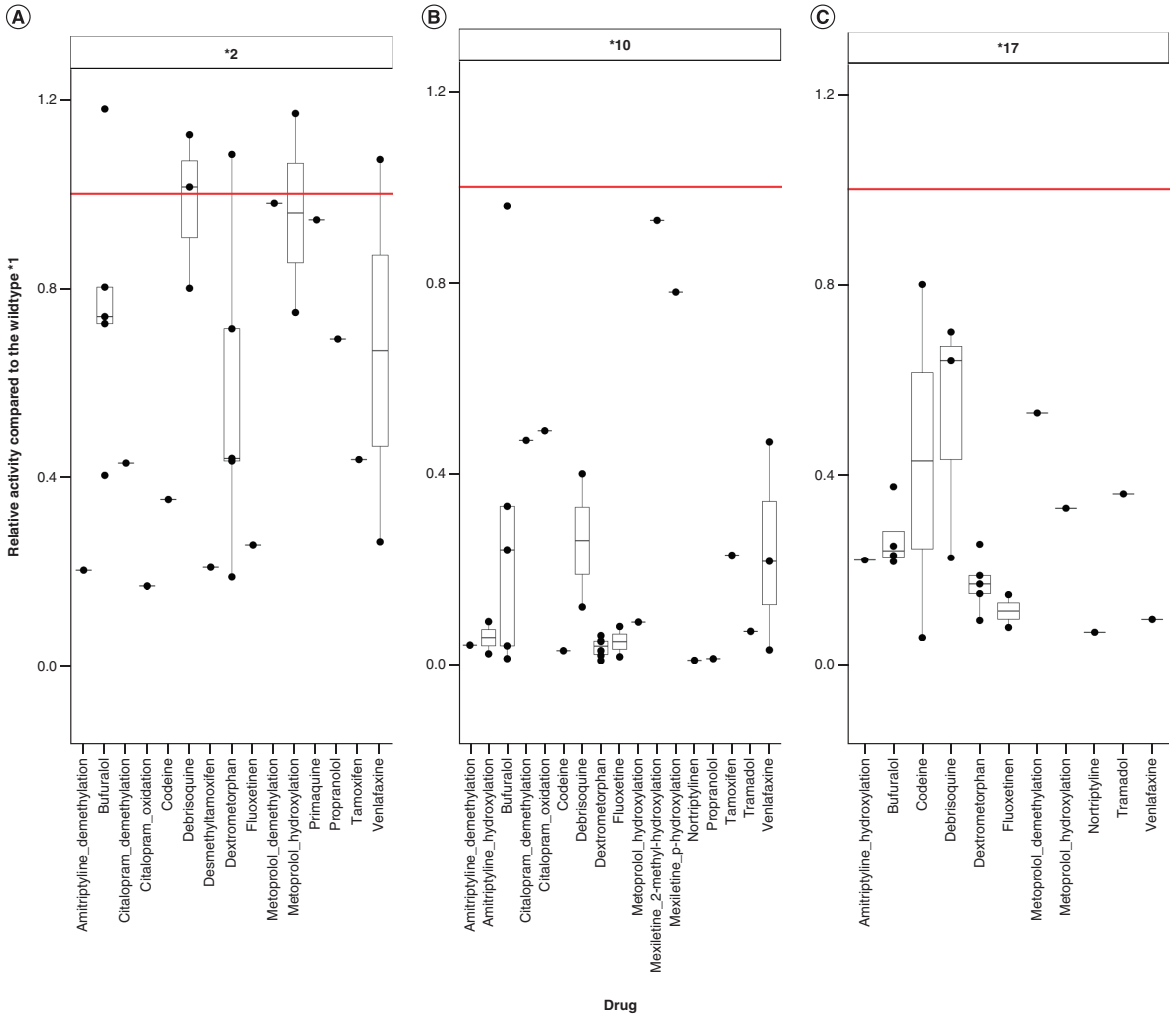

Figure 1. ***In vitro*** **activity of *2, *10 and *17.** Activity is calculated as the clearance of the allele of interest divided by the clearance of the wildtype. The red line represents the wildtype activity of 1.0. **(A)** The *2 activity for 13 substrates and 15 conversions. **(B)** The activity of *10 for 14 substrates and 17 metabolic steps. **(C)** The activity of the *17 allele for 11 substrates and 12 metabolic conversions.

For the common substrates, the metabolic activity of *CYP2D6**17 for debrisoquine is almost twice as high compared with the other substrates (Table 1 & Figure 1). When comparing findings within studies, two out of three studies report a similar trend (0.64 and 0.70 for debrisoquine and 0.04 and 0.25 for bufuralol; Figure 2 & Supplementary Table 1) [25,27]. By contrast, Marcucci *et al.* report an activity of 0.23 for debrisoquine compared with 0.22 for bufuralol and 0.19 for dextromethorphan when using baculovirus, indicating comparable activity for all three substrates [24]. This suggests a potential substrate-specific effect of *CYP2D6**17 resulting in a higher activity for debrisoquine.

Results for the other substrate were equally diverse. Most interestingly, the two studies investigating codeine O-demethylation reported both the highest activity of *CYP2D6**17 (0.8) [27] and the lowest activity (0.06) [28]. Both of these studies used the same type of vector, namely, a baculovirus. Moreover, only four observations reported an activity above 0.5, indicating a clear decreased activity function of *17 for all substrates.

Discussion

In this paper, we summarize available *in vitro* data regarding CYP2D6 substrate specificity. Our study supports the existence of substrate-specific effects of the *17 allele, a more subtle effect of the *10 allele and no substrate specificity of the *2 allele, in the metabolism of dextromethorphan, bufuralol, debrisoquine and venlafaxine. The activity of the *17 allele was higher for debrisoquine compared with bufuralol and dextromethorphan in two out

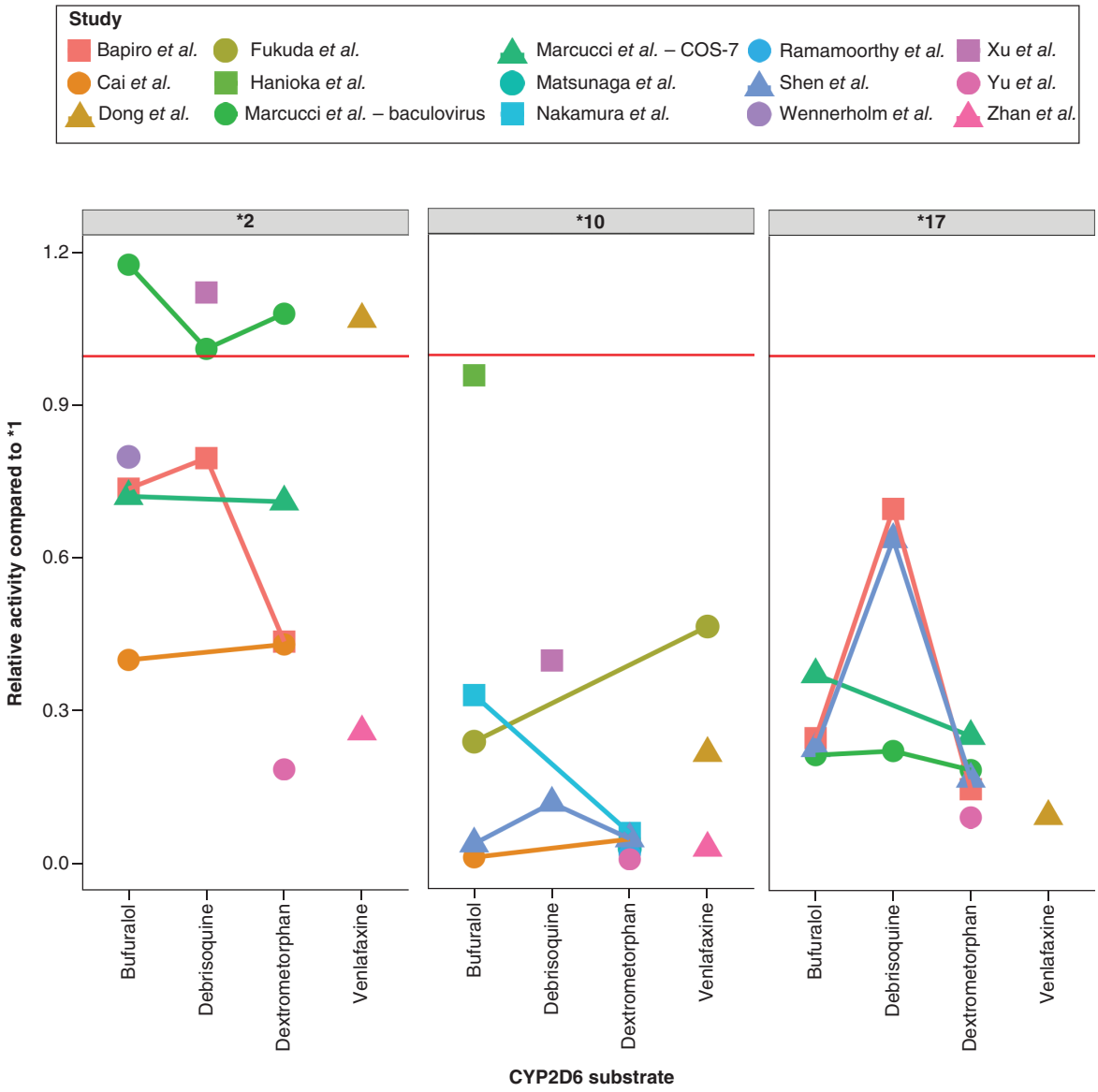

Figure 2. CYP2D6 substrate specificity. Within study comparisons for the commonly studies substrates; bufuralol, debrisoquine, dextromethorphan and venlafaxine. Each line connects different observations within the same study, thereby correcting for study specific effects. The majority of the studies show substrate-specific activities but are not in agreement with other studies. For CYP2D6*17 a clear substrate-specific effect for debrisoquine is observed.

of three studies; in the third study, the activity was comparable. Nonetheless, the variability of reported activities for the same substrate was large between studies, making it difficult to distinguish substrate-specific effects from study-specific effects. The variants included in the characterization of *17 are 2851C >T (R296C), 4181C >G (S486T) and 1022C >T (T107I). The first two variants are also the characteristic variants of the *2 allele; therefore, a comparison between *2 and *17 focuses on the contribution of the 1022C >T variant. The *17 allele was the first allele for which a substrate-dependent affinity was reported. As Oscarson *et al.* indicated, the 1022C >T mutation affected the metabolism of codeine but did not have a significant effect on the metabolism of bufuralol. To alter the metabolism of bufuralol, the combination of the 1022C >T and the 2851C >T was needed [29]. It has been suggested that the *17 allele is affecting protein binding by decreasing the substrate-access channel as well as a decrease in active sites of 21%. The effect of this decrease depends on the 3D structure and size of the substrate and thus results substrate-specific effects [22]. The relatively high activity of CYP2D6*17 in the metabolism of debrisoquine can potentially be explained by the size of the drugs. Debrisoquine is substantially smaller than the

other drugs at 175 g/mol for debrisoquine compared with 261 and 271 g/mol for bufuralol and dextromethorphan, respectively [30]. This might allow debrisoquine to still fit through the smaller substrate-access channel caused by the *CYP2D6**17 variant, whereas the other drugs do not fit as easily. Additionally, the 1022C >T mutation results in a change from the hydrophilic Thr to a hydrophobic Ile, right at the entrance of the substrate-binding channel causing alteration in hydrogen bonds and potentially in the attraction of substrates [22].

Interestingly, in the metabolism of debrisoquine the activity in each study was highest for *2. This might indicate the importance of the 2851C >T (R296C) and 4181C >G (S486T) variants in the increased activity of debrisoquine metabolism. Similar to the *17 allele, the *2 allele also results in a decrease in substrate-access channel albeit it is a smaller decrease compared with *17. It has been suggested that this smaller decrease results in a tighter binding pocket and therefore a better fit for some substrates [22].

The *CYP2D6**10 haplotype is characterized by 4181C >G (S486T) and 100C >T (P34S). the predicted activities for *10 differed greatly, with an activity of <0.1 for nine substrates and a group of six substrates with activities higher than 0.25. The low *in vitro* activity observed is in line with a previous review investigating *CYP2D6**10 substrate specificity. This review reported that the *10 allele decreased the CYP2D6 activity by more than 90% for most substrates, except for mexiletine and venlafaxine. Similar to the current study, it was also concluded that the large variability between studies complicates the interpretation and quantification of substrate-specific effects [16]. Several *in vitro* and *in vivo* studies have shown that the amount of functional protein expressed by *CYP2D6* *10 is many-fold lower than that of a wildtype allele [31–34]. Decreased levels of protein expression would mean that all substrates are similarly affected. Nonetheless, In addition to decreased expression, *CYP2D6**10 also leads to a smaller substrate-access channel, similar to *2 and *17 [22]. This change in active sites and substrate-binding channel combined with the decrease in expression can lead to a substrate-specific effect in addition to a general lower activity due to a decrease in expression. This could explain that while CYP2D6*17 has the largest changes in substrate-binding sites and access channel, it is *CYP2D6**10 that displays the lowest activity.

As previously mentioned, the heterogeneity between *in vitro* experiments is a known problem and can potentially be explained by multiple factors. A potential source of variability in results between studies could be the transfection vector used [16,19,20]. Indeed, studies using yeast cells reported slightly higher CYP2D6 activities compared with the other transfection vectors. Nonetheless, this effect was limited and was not observed for the other vectors. Moreover, for *CYP2D6**17 metabolism of codeine the highest and lowest activities (0.06 and 0.8) were reported by studies using the same vector, namely a baculovirus. Another cause for the observed heterogeneity between studies might be the genetic make-up of the *1 template used for the wildtype. Although some studies used human *CYP2D6**1 cDNA which was checked by sequencing [22–26,28,35], others reverse engineered cDNA of the variant of interest to serve as the wildtype comparator [33]. This means that there are potential genetic differences in the wildtype templates used by the different studies. Variants in the wildtype alleles can influence its activity and thereby the relative activity of the other alleles. Moreover, for *in vivo* experiments a suggested cause of variation in the activity of the *2 allele is the presence of an upstream enhancer [36,37]. However, no upstream variants are included in the applied *in vitro* assays and therefore the role of the suggested upstream enhancer cannot be assessed with *in vitro* experiments.

The majority of the current PGx guidelines relate to genetic variants associated with drug metabolism. As such, predicting *in vivo* enzyme activity based on genetic test results is instrumental for clinical PGx. Marcath *et al.* have shown that for *CYP2D6**10 the activity observed *in vitro* does not necessarily translate to the *in vivo* setting. More specifically, they noted that the decrease in CYP2D6 activity was approximately 90% in most *in vitro* studies. However, in the human PK studies this decrease was between 15.9% and 69.9%, which is substantially lower [16]. One of the main hurdles in translating *in vitro* results to an *in vivo* setting is the influence of variants of CYP2D6 protein expression levels. Protein expression can vary greatly between different transfection systems [18–20]. The parameters used in this study were all adjusted for protein content (V_{max} = pmol substrate/pmol CYP2D6) to cancel out the differences in regards to protein expression. However, by using this correction it is no longer possible to detect any changes in expression caused by the introduced variants.

Although our study provides insight into substrate specificity of CYP2D6 there were also several limitations. First and foremost, due to the heterogeneity between *in vitro* experiments, it was not possible to quantitatively compare findings between studies. For only a few substrates, there was sufficient data to allow within-study comparisons, thereby correcting for study-specific effects. Additionally, *in vitro* data in itself suffers from limitations when it comes to PGx effect predictions. Small differences in set-up (e.g., vector and origin of cDNA template) can result in

large differences in outcomes. Moreover, *in vitro* findings do not always translate to an *in vivo* prediction, especially not in the case of protein expression.

Conclusion

Our results support that genetic variation in *CYP2D6* show substrate-specific effects *in vitro*. Nonetheless, for many CYP2D6 substrates there is limited data regarding *in vitro* enzyme activity which complicates the study of substrate specificity. Moreover, *in vitro* data is limited in assigning accurate quantitative activity scores due to the high heterogeneity between studies.

Future perspective

Pharmacogenomics-informed drug prescribing is becoming standard clinical practice. However, current clinical guidelines assume that the effect of a particular genetic variant on metabolism is independent of the drug substrate. However, as our study has shown, there are clear substrate-specific effects that can be explained by specific variant-substrate interactions. To further improve our ability to predict drug response and thereby improve therapy outcomes, substrate-specific effects should be accounted for in the clinical pharmacogenomics guidelines.

Executive summary

- Data for four *CYP2D6**-haplotypes (*1, *2, *10, *17) was collected and compared. Four substrates (dextromethorphan, debrisoquine, bufuralol and venlafaxine) were studied in more than two studies, allowing for a more detailed analysis of the substrate-specific effects. Another 15 substrates were included in only 1 or 2 studies.
- There is significant variability in kinetic effects of the *CYP2D6* alleles dependent on the substrates, with differences in kinetic effects ranging from 1.02- to 5.4-fold for the same allele. Most significantly, the *17 allele showed a 4.7-fold higher activity for debrisoquine compared with the other drugs.
- On average, *CYP2D6**10 was the least active, followed by *CYP2D6**17 and *CYP2D6**2. For *CYP2D6**10 large differences in activity were observed with an activity of <0.1 for nine substrates and a group of six substrates with activities higher than 0.25. *CYP2D6**17 was more active in regards to debrisoquine metabolism compared with the other substrates. Finally, *CYP2D6**2 displayed the largest variation in activity ranging from loss of function at 0.17 to gain of function at 1.18.
- We observed high, unexplained, variability between studies resulting in an inability to unambiguously determine the impact of variants on absolute CYP2D6 activity and to quantify the effect of substrate specificity.
- Changes in protein-binding channels are potentially the cause of the substrate-specific effects. These changes result in an altered affinity of the substrate for the binding site that can differ per substrate depending on substrate characteristics. This indicates that substrate-specific effects originate at the variant level. However, current clinical guidelines only differentiate at the phenotype level.
- Our study confirms the existence of clear substrate-specific effects of the *CYP2D6**17 allele, a subtle effect of the *CYP2D6**10 allele and no substrate specificity of the *CYP2D6**2 allele, in the metabolism of dextromethorphan, bufuralol, debrisoquine and venlafaxine.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/suppl/10.2217/pgs-2021-0093

Author contributions

M van der Lee, H-J Guchelaar and J Swen wrote the manuscript. Literature search and interpretation were done by M van der Lee.

Financial & competing interests disclosure

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