BBA - General Subjects 1868 (2024) 130595

Contents lists available at ScienceDirect

BBA - General Subjects

journal homepage: www.elsevier.com/locate/bbagen

Uncoupling of Cytochrome P450 2B6 and stimulation of reactive oxygen species production in pharmacogenomic alleles affected by interethnic variability

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ARTICLE INFO

Keywords:
CYP2B6
CYP2B6*6
Pharmacogenomics
ROS
Uncoupling
Peroxide shunt

ABSTRACT

Cytochrome P450 mediated substrate metabolism is generally characterized by the formation of reactive intermediates. *In vitro* and *in vivo* reaction uncoupling, results in the accumulation and dissociation of reactive intermediates, leading to increased ROS formation. The susceptibility towards uncoupling and altered metabolic activity is partly modulated by pharmacogenomic alleles resulting in amino acid substitutions. A large variability in the prevalence of these alleles has been demonstrated in CYP2B6, with some being predominantly unique to African populations.

The aim of this study is to characterize the uncoupling potential of recombinant CYP2B6*1, CYP2B6*6 and CYP2B6*34 metabolism of specific substrates.

Therefore, functional effects of these alterations on enzyme activity were determined by quantification of bupropion, efavirenz and ketamine biotransformation using HPLC-MS/MS. Determination of H_2O_2 levels was performed by the AmplexRed/horseradish peroxidase assay.

Our studies of the amino acid substitutions Q172H, K262R and R487S revealed an exclusive use of the peroxide shunt for the metabolism of bupropion and ketamine by CYP2B6*K262R. Ketamine was also identified as a trigger for the peroxide shunt in CYP2B6*1 and all variants. Concurrently, ketamine acted as an uncoupler for all enzymes. We further showed that the expressed CYP2B6*34 allele results in the highest H_2O_2 formation.

We therefore conclude that the reaction uncoupling and peroxide shunt are directly linked and can be substrate specifically induced with K262R carriers being most likely to use the peroxide shunt and R487S carrier being most prone to reaction uncoupling. This elucidates the functional diversity of pharmacogenomics in drug metabolism and safety.

1. Introduction

The human Cytochrome P450 2B6 (CYP2B6) enzyme is involved in the metabolism of about 10% of all drugs and is known to have pharmacogenetic polymorphisms that vary in prevalence worldwide [52]. To date, 37 haplotypes have been identified, of which 19 result in a decreased or less-functional enzyme (PharmVar). Eleven of these haplotypes are characterized by a 785 A > G point mutation (K262R), ten of which appear together with the 516 G > T point mutation (Q172H). Unlike other CYP enzymes, CYP2B6 does not have null-alleles that lead to a complete loss of functionality.

The most prominent, low-activity allele is *CYP2B6*6* that additionally differs in prevalence from one ethnicity to another. For example the prevalence of *CYP2B6*6* is around 25% in Caucasians [17,25] and 40% in some African populations [2].

On account of this, in 2008 Nyakutira et al. [38] investigated the effects of the *CYP2B6*6* genotype on the CYP2B6 substrate efavirenz in a pharmacokinetic study in Zimbabwean HIV/AIDS patients. The study

Abbreviations: CYP450, Cytochrome P450; BUP, Bupropion; EFV, Efavirenz; KETA, Ketamine; HB, 6-Hydroxybupropion; HE, 8-Hydroxyefavirenz; NK, Norketamine.

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https://doi.org/10.1016/j.bbagen.2024.130595
Received 4 December 2023; Received in revised form 29 February 2024; Accepted 4 March 2024
Available online 10 March 2024

revealed that the application of the drug label recommended dose led to efavirenz plasma concentrations far above the recommended therapeutic range in *CYP2B6*6* carriers. The effect was more pronounced in women, showing generally high efavirenz levels irrespective of the genotype. These alterations lead to increased side effects and non-adherence to the treatment [30]. Based on these information and to increase drug safety, specific dosing recommendation guidelines have been worked out by the *Clinical Pharmacogenetics Implementation Consortium* (CPIC®) [9].

Though *CYP2B6*6* is commonly accepted to be a loss-of-function allele, *in vivo* and *in vitro* studies have investigated the enzyme's functionality, with versatile outcomes.

Most *in vivo* studies showed altered pharmacokinetics: While elevated plasma concentrations of efavirenz and ketamine [4,27,29,44] and decreased hydroxybupropion formation [15,22,24] were observed, the CYP2B6 substrate cyclophosphamide showed increased clearance in *CYP2B6*6* carriers [37].

In the context of ketamine metabolism, two separate *in vivo* studies, conducted by Li et al. [27] and Rao et al. [40], yielded discordant outcomes. Li et al. observed decreased clearance in *CYP2B6*6* carriers, whereas Rao et al. found no discernible difference in ketamine metabolism based on *CYP2B6* genotype. Consequently, the current state of available data for ketamine metabolism remains ambiguous.

In vitro CYP2B6*6 studies validated most *in vivo* findings: decreased substrate affinities towards bupropion, ketamine [26] and – to a higher extent – efavirenz [49] and increased catalytic ability in cyclophosphamide [48] metabolism were found using human liver microsomes.

*CYP2B6*6* harbours a double amino acid substitution, K262R (*CYP2B6*4*) and Q172H. Bumpus et al. [8] studied CYP2B6*4 and found increased efavirenz metabolism. To determine the effects of the double substitution resulting in CYP2B6*6 phenotype, Ariyoshi et al. [1] compared the efavirenz and cyclophosphamide metabolic activity of CYP2B6*4 and CYP2B6*6 and found a 50% decrease in efavirenz metabolism by CYP2B6*6 and an increased metabolism by CYP2B6*4. In contrast, cyclophosphamide metabolism was decreased in CYP2B6*4 and increased in CYP2B6*6 enzymes with a 60% reduction in K_m value compared to the wild-type.

Thus, K262R is hypothesized to have an inverse effect on the metabolic activity of CYP2B6 compared to Q172H, which seems to abolish effects of K262R in efavirenz and cyclophosphamide metabolism of CYP2B6*6.

1459C > A (R487S) is a further exonic variant with an amino acid exchange that was described to co-occur with the variants of CYP2B6*6. The resulting allele was termed *CYP2B6*34* and encodes the three exonic SNPs 516 G > T, 785 A > G and 1459C > A and a promoter-located SNP that decreases the gene expression. This allele has so far only been assigned to African populations with a frequency of 0.12% [18,39,47]. While the two substitutions K262R and Q172H were intensely studied, the effects of the third amino acid exchange in combination (R487S substitution) remain largely unknown.

One isolated *in vitro* study on this substitution was performed by Radloff et al. and demonstrated that the R487S substitution results in an increased specific activity towards efavirenz and bupropion. Based on this observation and the extensive characterization of CYP2B6*6, Radloff et al. postulated a wild-type like activity of the CYP2B6*34 enzyme, even though the promoter variant is expected to decrease the protein content. This effect however can't be explained by structural malformations since the positions of the three amino acid substitutions neither affect the substrate binding or the active centre nor the POR binding sequence [7] or the membrane anchor domain [13].

Given that, the explanation for substrate-dependent variability in enzyme function may involve non-structural mechanisms. A functional deviation that has been associated with amino acid exchanges of other human and non-human CYP450 enzymes is reaction uncoupling [3,6,14,28,50].

Reaction uncoupling is defined as a dissociation of two cascaded reaction steps without substrate oxidation.

Usually, the CYP450 reaction cycle comprises nine steps including the formation of several reactive intermediates that accumulate in case of a reaction uncoupling event. Due to their unstable nature, dissociations are likely and result in increased ROS levels. The reverse reaction is called peroxide shunt and describes the use of H_2O_2 as a reaction driver [36]. H_2O_2 however is known to deactivate enzymes by thiol group oxidation [5] and oxidation of heme groups, resulting in a decreased H_2O_2 tolerance of CYP450 enzymes [21]. The use of the peroxide shunt is further limited by a decreased reaction efficiency [31]. Hence both reactions result from and in altered coupling efficiences.

Since the binding direction of substrates within the binding pocket has been shown to impact coupling efficiency [16], predictions on uncoupling susceptibility often rely on altered amino acid properties at active protein sites, hence substrate binding site, reaction center or, if present, allosteric binding sites.

While this information is valuable and allows for computational approaches, observations from our work and others indicate that single amino acid substitutions at sites that are not directly involved in enzymatic activity (non-active protein sites) may increase uncoupling of CYP2C8*2, CYP2C8*3 [50], CYP2C9*2, and CYP2C9*3 [41,46]. These residue changes should not affect substrate binding or metabolism but may decrease the coupling efficiency in the substrate metabolism of the affected enzymes.

The aim of this study was to explore the effect of the amino acid exchanges observed in the CYP2B6*6 and CYP2B6*34, that all lay outside the substrate binding region or active site of the enzyme. The enzymes will be studied with regard to functional effects that could explain the observed variability in substrate specific oxidation by CYP2B6. Therefore, bupropion, efavirenz and ketamine were used as substrates.

Our results will increase the understanding of the functional specificities that may affect substrate turnover and efficiency of the oxidative reaction of the different variants.

2. Methods

2.1. Generation of CYP2B6*1, CYP2B6*6 and CYP2B6*34 encoding plasmids

pET28a(+) plasmid encoding *CYP2B6*1* cDNA was purchased from Genscript (NM_000767) and the SNPs characteristic for *CYP2B6*34* and *CYP2B6*6* were introduced by site directed mutagenesis PCR using the Phusion® High-Fidelity DNA Polymerase mix (*New England Biolabs,* M0530L). Plasmids encoding the SNP combinations to express *CYP2B6*6* and *CYP2B6*34* as well as the single nucleotide polymorphisms alone were produced for recombinant expression. The respective forward and reverse primer sequences are ACATACCAGATCAGCTTCCT and AAGCTCATCTGGTATGTTGG for the introduction of 1459C > A, TTCCATTCCATTACCGCCA and GGAAGGAAGAGGAAGGT for the introduction of 516 G > T and CCAGGGACCTCATCGACA and GTCCCTGGGGGCGCTG for the introduction of 785 A > G.

Successful PCR product was verified by transformation into *Escherichia coli* (*E. coli*) post DPNI digest (*New England Biolabs,* R0176S). Grown single colonies were picked and cultured allowing isolation of variant plasmids. To ensure correct SNP introduction, TubeSeq sanger sequencing services provided by *Eurofins Genomics* were used.

2.2. Transformation of Escherichia coli

NADPH-CYP450-Oxidoreductase (POR) encoding pET-22b(+) plasmid and *CYP2B6*1, CYP2B6*6* and *CYP2B6*34* as well as the single amino acid variants Q172H, K262R and R487S encoding pET-28a(+) plasmids were transformed into competent C41 *Escherichia coli* (*New England Biolabs*) via a heat-shock of 45 s at 42 °C. The cells were chilled

on ice for a few minutes and resuspended in lysogeny broth (LB) medium (*Roth*) supplemented with 2.5 mM KCl, 5 mM $MgCl_2$, 10 mM $MgSO_4$ and 25 mM glucose. The suspension was incubated for at least 1 h at 37 °C before being plated on selection agar plates. Agar plates were incubated over night at 37 °C. The next day, single colonies were picked and cultured for recombinant enzyme production.

2.3. Recombinant enzyme expression

Bacterial culture was started at an OD_{600} of 0.08 and grown at 37 °C in LB- Medium containing the respective antibodies. At an OD_{600} of 0.8–1.2, plasmid expression was induced with 0.1 mM IPTG and δ-aminolevulinic acid was added to the CYP expressing bacteria. The cultures were incubated at 30 °C over night. After harvest by centrifugation at 1500 *xg* for one hour, the cells were resuspended in 100 mg/mL 250 mM sucrose in $_{dd}H_2O$, protease inhibitor cocktail (*MedChemExpress*, HY-K0011), phosphatase inhibitor cocktail II (*MedChemExpress*, HY-K0022), phosphatase inhibitor cocktail III (*MedChemExpress*, HY-K0023), 100 μM PMSF, 45 mM cysteine, 4 mM DTT and 300 μM phosphocholine. Cell lysis was performed by 6 × 30 s sonication on ice with an amplitude of 20% and a one minute break between each sonication step.

2.4. Microsome preparation

Enzyme preparation was performed via differential centrifugation of the cell lysate. In a first 10 min long step at 5000 *xg*, cell debris and nuclei were removed from the suspension. The resulting supernatant was further centrifuged at 10,000 *xg* for 20 min. The final 1 h long ultracentrifugation step was performed at 50,000 *xg* in the Optima LE-80 K Ultracentrifuge (Beckmann Coulter) using the fixed angle rotor Ti 70.1 (*Beckmann,* No.: 342184).

To determine the integrity of the expressed enzymes, CO spectroscopy was performed with all CYP2B6 constructs as established by Guengerich et al. [12]. Both absorptions at 420 nm and at 450 nm were determined and the respective concentrations were calculated as described below. As a control, empty bacteria without plasmids were assessed and the resulting values were subtracted from the CYP450 micrsomal preparations. Fig. 1 shows the corrected concentrations. Accordingly, all constructs were expressed and the resulting enzymes were viable at similar concentrations.

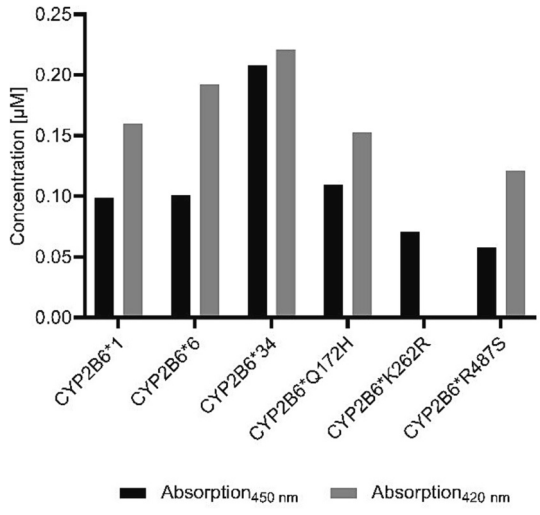

Fig. 1. CO-spectroscopy results of microsomal fractions isolated from *Escherichia coli* expressed human CYP2B6 and CYP2B6 variants. CYP2B6*1, CYP2B6*6, CYP2B6*34, CYP2B6*Q172H, CYP2B6*K262R, CYP2B6*R487S enzymes were expressed in *E. coli* and isolated as described. The respective preparations were tested on their integrity via absorption at 450 nm.

2.5. Determination of POR and CYP concentration in enzyme solution

To determine the amount of active enzyme in solution, the spectrometric approach described by Guengerich et al. [12] was applied. CYP containing solutions were first mixed with CO gas and the base absorption at 400 nm – 500 nm was measured. $Na_2S_2O_4$ was added to the solution in order to reduce the ferric ion to a ferrous ion, allowing the binding of CO and the associated characteristic absorption peak shift to 450 nm. In order to calculate the amount of active CYP enzyme, the following formula is applied to the absorption values at 450 nm and 490 nm:

$$\frac{A_{450} - A_{490}}{0.91\ mM^{-1}\ cm^{-1}} \div 1cm^{*}Dilution\ factor = active\ CYP\ [mM] \quad (1)$$

POR activity can be determined via its NADPH-cytochrome c reduction activity. Therefore, 80 μL of 0.5 μM horse heart cytochrome *c* is added to 1 mg of the POR solution. A baseline is recorded over a few minutes at 550 nm. Fresh NADPH is added to a final concentration of 0.1 mM and the recording is continued for further three minutes. To calculate the reduction activity of the POR sample, the following formula is applied:

$$\frac{\Delta A_{550}}{time\ [min]} \div 0.021\ mM^{-1}cm^{-1} = [Cytochrome\ C\ reduced\ in\ sample] \quad (2)$$

2.6. Determination of CYP2B6 activity

The reaction was set up in 250 mM sucrose in $_{dd}H_2O$, protease inhibitor cocktail (*MedChemExpress*, HY-K0011), phosphatase inhibitor cocktail II (*MedChemExpress*, HY-K0022), phosphatase inhibitor cocktail III (*MedChemExpress*, HY-K0023), 100 μM PMSF, 45 mM cysteine, 4 mM DTT, 300 μM phosphocholine. In all reactions, a final concentration of 0.01 μM CYP2B6 enzymes and 193 nmol cytochrome *c* reduced/min of POR stock were applied. Microsomal enzymes were incubated with 10 μM bupropion, efavirenz or ketamine and 100 μM NADPH at 37 °C for 1 h. The incubation was stopped by addition of two volumes ice cold acetonitrile. To eliminate protein residues, the denatured samples were centrifuged for 45 min at 3220 x*g* and 4 °C. The resulting supernatant was collected and evaporated under vacuum to concentrate the sample. The resulting sample was solubilized in 40% acetonitrile in water and substrate and metabolite concentrations were determined via HPLC-MS/MS.

2.7. Determination of extracellular H_2O_2 via Amplex Red oxidation to resorufin

For the final reactions, 0.01 μM CYP2B6 enzymes and 193 nmol cytochrome *c* reduced/min of POR stock were applied. Microsomal enzymes were incubated with 10 μM bupropion, efavirenz or ketamine and 100 μM NADPH at 37 °C for 1 h. The resulting medium was removed and assayed via the Amplex Red assay. The assay is based on the conversion of Amplex Red by a horseradish peroxidase in the presence of hydrogen peroxide (H_2O_2) into the fluorescent product resorufin, with a 1:1 stoichiometry to H_2O_2 consumption, allowing for quantification via photospectrometry [34]. The set up consisted of the reaction mix containing CYP2B6 enzymes, POR, NADPH and the respective substrates. A control with buffer only was always included to subtract the background fluorescence without reaction from each reaction well. The assay was established along with an empty bacterial strand to exclude other microsomal activity. The possible autoxidation of NADPH was tested by assaying the microsomal fractions with and without NADPH. All results of the assay validation are depicted in Fig. 2. Overall, the assay was found to be suitable since no control reaction showed positive outcomes within the reaction range. The autoxidation of NADPH can be excluded

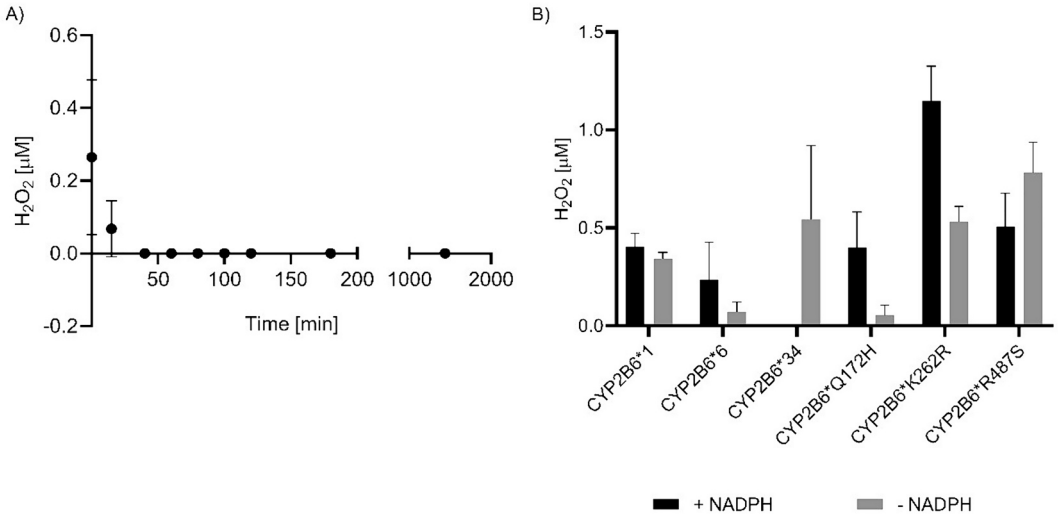

Fig. 2. Validation of the Amplex Red/horseradish peroxidase assay for determination of H_2O_2 production in CYP2B6 reactions. A) Empty bacterial strand samples were used for microsome reactions and the H_2O_2 production was assayed over 24 h. B) H_2O_2 production of microsomal preparations from recombinant CYP2B6*1, CYP2B6*6, CYP2B6*34, CYP2B6*Q172H, CYP2B6*K262R, CYP2B6*R487S enzymes with and without NADPH to assess NADPH autoxidation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

since the samples with NADPH did not show higher H_2O_2 concentrations compared to the samples without NADPH.

For H_2O_2 quantification, a standard curve, of 10 µM, 5 µM, 2.5 µM, 1.25 µM, 0.63 µM, 0.31 µM, and 0.16 µM H_2O_2 (Sigma, 386,790-100ML-M) was prepared in 50 µL reaction buffer. The 50 µL standard samples were transferred into a black, opaque 96-well plate.

H_2O_2 concentrations were determined after the reaction incubation time by an Amplex Red (AR (Sigma, 90,101-5MG-F)/horseradish peroxidase (Sigma, P8375-2KU) mix (AR/HRP mix) using final concentrations of 25 µg/mL AR and 0.2 U/mL HRP. The mix was freshly prepared before each experiment. The reaction was initiated by combining 50 µL of the AR/HRP mix with 50 µL of prepared standard and CYP samples. After a 30-min incubation at room temperature, the fluorescence of resorufin ($A_{exitation\ =}$ 570 nm / $A_{emission\ =}$ 590 nm) was measured using a plate reader. H_2O_2 concentrations in the CYP samples were determined by applying linear regression on the standard curve values.

2.8. Determination of K_M and V_{max} values for efavirenz, bupropion and ketamine binding to CYP2B6

Substrate binding affinity was classified by means of *Michaelis-Menten* kinetics. Thereto, recombinant CYP2B6 and the respective enzymatic variants were used. Substrate concentrations ranging from 1 µM to 100 µM were applied and the catalyses were performed over 120 min in 250 mM sucrose in $_{dd}H_2O$, protease inhibitor cocktail (*MedChemExpress*, HY-K0011), phosphatase inhibitor cocktail II (*MedChemExpress*, HY-K0022), phosphatase inhibitor cocktail III (*MedChemExpress*, HY-K0023), 100 µM PMSF, 45 mM cysteine, 4 mM DTT, 300 µM phosphocholine. The reaction was stopped and the samples were processed as described above. Measurements of substrate concentrations were performed by HPLC-MS/MS.

2.9. Nomenclature

In this study, we explore the impact of amino acid substitutions Q172H, K262R, and R487S, and their corresponding enzyme variants CYP2B6*6 (Q172H, K262R) and CYP2B6*34 (Q172H, K262R, R487S). The nomenclature for single amino acid variants adheres to standard allele assignment conventions, leading to designations like CYP2B6*Q172H, CYP2B6*K262R, and CYP2B6*R487S. Notably, while CYP2B6*K262R aligns with the established CYP2B6*4 [25] allele, we have opted to maintain a distinct nomenclature. This decision is deliberate, as it emphasizes our primary focus on K262R within the broader context of the CYP2B6*6 and CYP2B6*34 phenotypes.

2.10. HPLC-MS/MS settings

Hydroxylation of efavirenz, bupropion and *N*-demethylation of ketamine was determined by HPLC-MS/MS. The system consisted of a SCIEX QTRAP 6500+ (*AB Sciex*) triple quadrupole mass spectrometer coupled to an Agilent 1290 Infinity II UHPLC. Chromatographic analyte separation was performed on a 2.1 × 50 mm Poroshell phenyl-hexyl column with a pore size of 2.7 µm (*Agilent Technologies*) for bupropion and efavirenz and a 3.0 × 100 mm Accucore C18 with 2.6 µm pore size for ketamine (*Thermofisher Scientific)*.

The chromatographic separation of substrates and metabolites was based on a gradient of 0.1% (*v*/v) formic acid in water and 0.1% (v/v) formic acid in acetonitrile for bupropion, efavirenz and their metabolites or methanol for ketamine and norketamine. 6-Hydroxy−/bupropion and 8-hydroxy−/efavirenz gradient separation ran with a flow rate of 0.6 ml/min. Nor−/ketamine separation was performed on a flow rate of 0.4 ml/min.

The gradient to separate bupropion and hydroxybupropion started with 95% of 0.1% formic acid in water for the first 1.5 min. The amount of 0.1% formic acid in acetonitrile was slowly increased to 98% over 1.5 min and left running until 4.4 min. A fast drop to 5% 0.1% formic acid in acetonitrile followed until 4.5 min and was kept until the end at 6 min.

To separate efavirenz and hydroxyefavirenz, the gradient was adjusted to 90% of 0.1% formic acid in water over one min and then slowly decreased to 10% of 0.1% formic acid in water until 4 min. The flow was kept at this rate until 4.4 min before being brought back to 90% of 0.1% formic acid in water over 1 min until the end at 7 min.

Ketamine and norketamine separation was performed with a gradient of 0.1% formic acid in water and 100% methanol. The gradient started with 95% of 0.1% formic acid in acetonitrile until 2.5 min, followed by an increase of methanol to 95% until 3.4 min. The gradient ran until 4.4 min before a fast switch to 95% of 0.1% formic acid in water which was kept until the end at 6 min.

Subsequent MS electrospray ionization was performed in a positive mode for bupropion and ketamine and in a negative mode for efavirenz.

Transitions of substrate and metabolite precursor ions to product

ions were determined to be *m/z* 240.7 to *m/z* 184 for bupropion, *m/z* 256.7 to *m/z* 238 for hydroxybupropion, *m/z* 314 to *m/z* 244 for efavirenz, *m/z* 330 to *m/z* 258 for hydroxyefavirenz, *m/z* 238 to *m/z* 125 for ketamine and *m/z* 224 to *m/z* 125 for norketamine.

Acquisition data were processed by means of the Sciex software Analyst version 1.7.2 and the companion software Sciex OS version 2.1.

Calibration curves were designed to cover a range of 0–10 μM of each substrate and metabolite. The steps were set at 0 μM, 0.6 μM (90% accuracy), 1.25 μM (103% accuracy), 2.5 μM (100% accuracy), 5 μM (100% accuracy), 10 μM (102% accuracy). The peaks were integrated by the companion software and the areas under the curve (AUCs) were determined in a timeframe of ±30 s of the identified peak. Relative quantification of metabolite to substrate was performed based on the AUC values.

2.11. Statistical data analyses

Parametrical test methods were applied for statistical data analysis. First variance equality was verified by application of a F-test followed by an unpaired, two-tailed *t*-test. Statistically significant differences of more than two groups were assessed by an ordinary on-way ANOVA. K_M and V_{max} values were determined by nonlinear fitting of dc/dt values. All data are presented as mean ± SD and were calculated from at least two independent experiments. The exact number of replicates is stated in the respective figure description. $P < 0.05$ was considered significant and differences were indicated by the respective *p*-value. All statistical analyses were performed with GraphPad Prism 9.4.1.

3. Results

Since both, clinical and *in vitro* data reported substrate-specific differences of CYP2B6 enzyme activity associated with the CYP2B6*6 allele, the aim of this study was to uncover the biochemical mechanism behind this inconsistent effects. Enzymes carrying the alleles *CYP2B6*1* (wild-type), *CYP2B6*6* and *CYP2B6*34* were expressed in a recombinant system and the three catalytic main pathways of enzyme uncoupling, peroxide shunt pathway and the main monooxygenase pathway were studied in all allelic enzyme variants.

3.1. Substrate turnover in recombinant CYP2B6*1, CYP2B6*6 and CYP2B6*34 enzymes

Substrate turnover was studied for each probe drug (bupropion, efavirenz, ketamine) in each variant using recombinant CYP2B6*1, CYP2B6*6 and CYP2B6*34 enzymes from transformed *E. coli.* To assure the enzymatic function and integrity, substrate turnover and binding affinities were determined.

Quantitative enzymatic metabolism was determined in each of the expressed allelic variants for the three substrates to gain full understanding of the respective phenotype. Since different metabolites can be formed at the same time by the enzyme, we measured the decline in substrate concentration instead of single metabolite formations. In addition, substrate binding affinities of all three substrates were experimentally determined as K_M values (Table 1).

As shown in Fig. 3, the largest difference in substrate turnover was seen between the CYP2B6*6 variant and wildtype, where bupropion turnover was higher (83.0% ± 4.6%) compared to CYP2B6*1 (30.0% ± 11.6%). In contrast, bupropion turnover was lowest in the CYP2B6*34 enzymatic variant (17.5% ± 6.7%). This observation in turnover was not reflected by the binding affinities of bupropion, where the wild-type showed the highest affinity (31 μM) followed by CYP2B6*6 (99 μM) and CYP2B6*34 (220 μM) (Table 1).

A similar lack of correlation between K_M and substrate turnover was seen in ketamine metabolism, in which CYP2B6*34 showed similar turnover (23.4% ± 0.5%) compared to CYP2B6*1 (26.5% ± 0.5%) and CYP2B6*6 (27.1% ± 0%) (Fig. 3) in spite of an undeterminably low binding activity of ketamine to CYP2B6*34 (Table 1).

In contrast, efavirenz turnover correlated positively with the binding affinities: CYP2B6*1 showed highest efvairenz turnover and binding affinity (55.7% ± 8.5%; K_M: 22 μM), followed by CYP2B6*34 (33.6% ± 1.4%; K_M: 113 μM) and CYP2B6*6 (66.1% ± 0%; K_M: 129 μM) (Fig. 3, Table 1).

Due to the opposing trends of ketamine and bupropion binding affinities and turnover in CYP2B6*6 and CYP2B6*34 mediated metabolism, the effect of the single amino acid substitutions CYP2B6*Q172H, CYP2B6*K262R and CYP2B6*R487S were studied separately in recombinant enzymes.

3.2. Functional impact of single amino acid substitutions Q172H, K262R and R487S in CYP2B6*6 and *34

Based on the K_M values, the combined amino acid substitutions seem to negatively affect substrate affinity compared to the wild-type. In order to adequately compare the effect of the single amino acid substitutions, the specific formation of 6-hydroxybupropion (HB), 8-hydroxyefavirenz (HE) and norketamine (NK) was quantified in enzymes carrying only one of the amino acid substitutions (Fig. 4).

Again, bupropion and ketamine metabolism appeared to be mostly affected by the amino acid exchange, whereas efavirenz metabolism was similar in all three variants and the wild-type.

Looking at the bupropion metabolism, in each of the two amino acid substitutions that account for the CYP2B6*6 genotype, significantly higher HB formation was achieved by CYP2B6*Q172H (10.9 ± 0.01 μM) compared to CYP2B6*K262R with only 0.3 ± 0.002 μM. Comparing all three variants and the wild-type, CYP2B6*1 showed highest HB formation (13.8 ± 0.03 μM). CYP2B6*R487S shows the lowest bupropion (6.6 ± 0.02 μM) and ketamine (0.9 ± 0.5 μM) metabolism of all enzymes. The drastic turnover increase observed in the CYP2B6*6 enzyme carrying both amino acid variants (Fig. 3) was not reproducible in the single amino acid substitutions.

Similarly, ketamine metabolism of CYP2B6*6 resulted in a wild-type like turnover (Fig. 3), while each single substitutions showed significantly decreased metabolite formation (Fig. 4).

CYP2B6*K262R shows the no ketamine metabolism with 0.05 ± 0.07 μM NK compared to 15.3 ± 0.7 μM in the CYP2B6*Q172H reaction and 21.5 ± 1.1 μM in the CYP2B6*1 reaction.

Table 1
K_M, V_{max} and catalytic efficiency (V_{max}/K_M) of bupropion, efavirenz and ketamine metabolism of recombinant CYP2B6*1, CYP2B6*6 and CYP2B6*34. Substrate concentrations were 10 μM in turnover determination and ranged from 1 μM to 100 μM in K_M assessment with $n = 2$. The reaction was stopped after 120 min. Nonlinear fitting of dc/dt values ± SD to the Michaelis-Menten equation allows determination of K_M values. Substrate turnover was determined using HPLC-MS/MS. First variance equality was verified by application of an F-test followed by an unpaired, two-tailed *t*-test. GraphPad Prism 9.4.1 was used for statistical analyses.

	CYP2B6*1				CYP2B6*6				CYP2B6*34			
	K_M [μM]	CI 95%	V_{max} [μM/min]	V_{max}/K_M	K_M [μM]	CI 95%	V_{max} [μM/min]	V_{max}/K_M	K_M [μM]	CI 95%	V_{max} [μM/min]	V_{max}/K_M
BUP	31	[22, 47]	11.6	0,025	99	[67, 155]	1,41	0,0143	220	[78, 259]	0,17	0,00076
EFV	22	[8, 55]	8.5	0,003	129	[21.2, +∞]	0,50	0,0038	113	[31 + ∞]	0,06	0,00061
KET	79	[58, 112]	0.45	0,001	105	[79, 145]	0,03	0,0003	N/A	N/A	0,17	0,00076

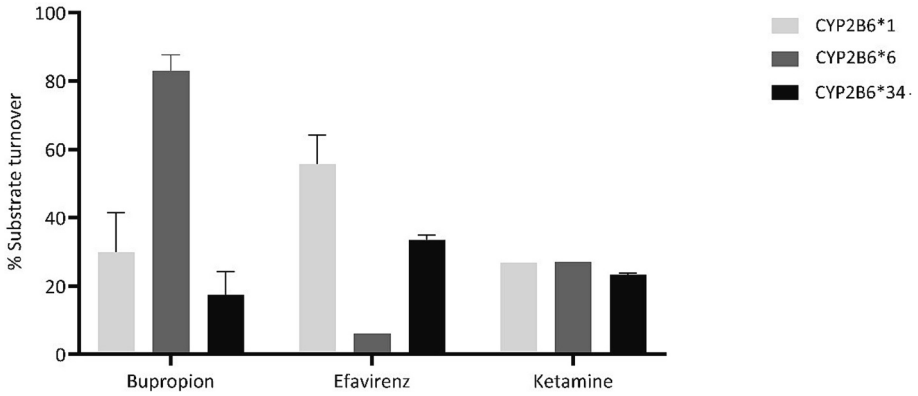

Fig. 3. Substrate turnover of bupropion, efavirenz and ketamine. Recombinant CYP2B6, CYP2B6*6 and CYP2B6*34 enzymes were incubated with 10 μM substrate for 1 h with a biological sample number of 2 per reaction. First variance equality was verified by application of an F-test followed by an unpaired, two-tailed *t*-test. All statistical analyses were performed with GraphPad Prism 9.4.1.

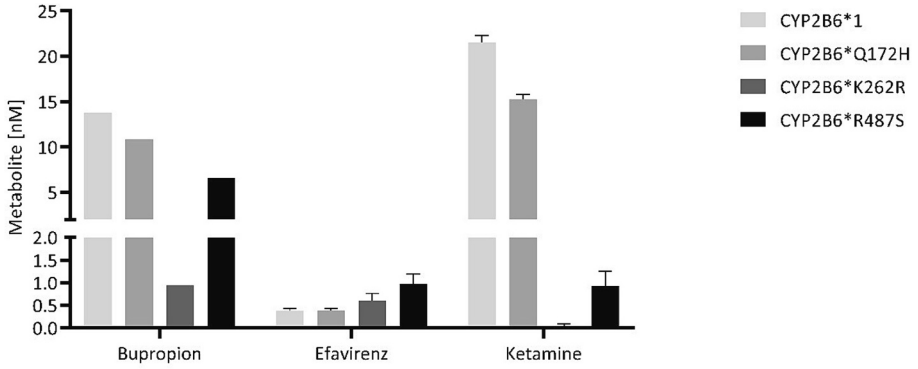

Fig. 4. 6-Hydroxybupropion, 8-hydroxyefavirenz and norketamine formation by CYP2B6*1, CYP2B6*Q172H, CYP2B6*K262R, CYP2B6*R487S after 1 h. 10 μM bupropion, efavirenz and ketamine were incubated for 1 h with each enzyme. Reaction was stopped and metabolite formation was determined by HPLC-MS/MS. First variance equality was verified by application of an F-test followed by an unpaired, two-tailed *t*-test with $n = 2$. All statistical analyses were performed with GraphPad Prism 9.4.1.

These results indicate that the two single amino acid substitutions do not account for the CYP2B6*6 phenotype in a simple additive manner.

The addition of R487S to CYP2B6*6 mirrors the CYP2B6*34 phenotype. The combination of all three substitutions resulted in the lowest binding affinities in all three substrates, while the turnover was only slightly reduced compared to CYP2B6*1.

Studying the single amino acid substitution R487S separately, metabolite formation was significantly decreased in bupropion and ketamine metabolism. Though still higher in bupropion and ketamine turnover than the CYP2B6*K262R variant, the R487S variant was less active than the CYP2B6*Q172H variant.

In the case of efavirenz, increased substrate turnover was already seen in the CYP2B6*34 variant. While not significantly different from the wildtype, R487S alone showed higher turnover of efavirenz (1.0 ± 0.3 μM) compared to CYP2B6*1 (0.4 ± 0.1 μM) and the other two amino acid substitutions (Q172H: 0.4 ± 0.1 μM; K262R: 0.6 ± 0.2 μM) (Fig. 4).

Taken together, the reason for the observed high turnover rates of the CYP2B6 alleles even at very low binding affinities cannot be explained by an additive effect of the single amino acid substitutions but may rather be grounded in different reaction pathways of the enzyme. Since the reaction cycle catalyzed by CYP450 enzymes can also proceed through the peroxide shunt pathway, we further investigated the ability of the three single amino acid substitutions to utilize hydrogen peroxide as a reaction driver.

*3.3. Use of shunt pathway (H_2O_2) for substrate oxidation of CYP2B6*1, CYP2B6*Q172H, CYP2B6*K262R and CYP2B6*R487S*

In CYP450 mediated reactions, H_2O_2 can be used as a reaction driver by directly converting the substrate bound, high-spin heme iron into the ferric-hydroperoxo species (compound 0) thereby bypassing the two single electron donations. The extent of peroxide shunt reactions is assumed to be inconsistent throughout the CYP450 enzymes. Experimentally, addition of H_2O_2 as the sole reaction driver in a biocatalysis reaction allows the assessment of the peroxide shunt usage for the enzyme-substrate couple.

Fig. 5 shows the results of H_2O_2 addition as a reaction driver, mediating oxidation of bupropion (Fig. 5A-D), efavirenz (Fig. 5E-H) and ketamine (Fig. 5I-L) by CYP2B6*1, CYP2B6*Q172H, CYP2B6*K262R and CYP2B6*R487S. Increasing concentrations of H_2O_2 were studied (1–100 μM) as the reaction driver of the peroxide shunt. Metabolism of the three substrates by CYP2B6*1, CYP2B6*Q172H, CYP2B6*K262R and CYP2B6*R487S *via* NADPH was used as a control to assess the use of the main pathway for substrate oxidation.

While efavirenz metabolism was again unaffected by the enzyme variant or the reaction driver, ketamine and bupropion metabolism was favourably executed with 10 μM H_2O_2 in CYP2B6*K262R and CYP2B6*R487S. Especially in CYP2B6*K262R, metabolism of bupropion resulted in a five-fold increase of metabolite formation with 10 μM H_2O_2 compared to NADPH (5.2 ± 1.0 nM HB *versus* 0.9 ± 0.002 nM). Ketamine metabolism of CYP2B6*K262R showed an even higher effect of H_2O_2 with 6 nM NK with 10 μM and 100 μM H_2O_2 compared to 0.1 ±

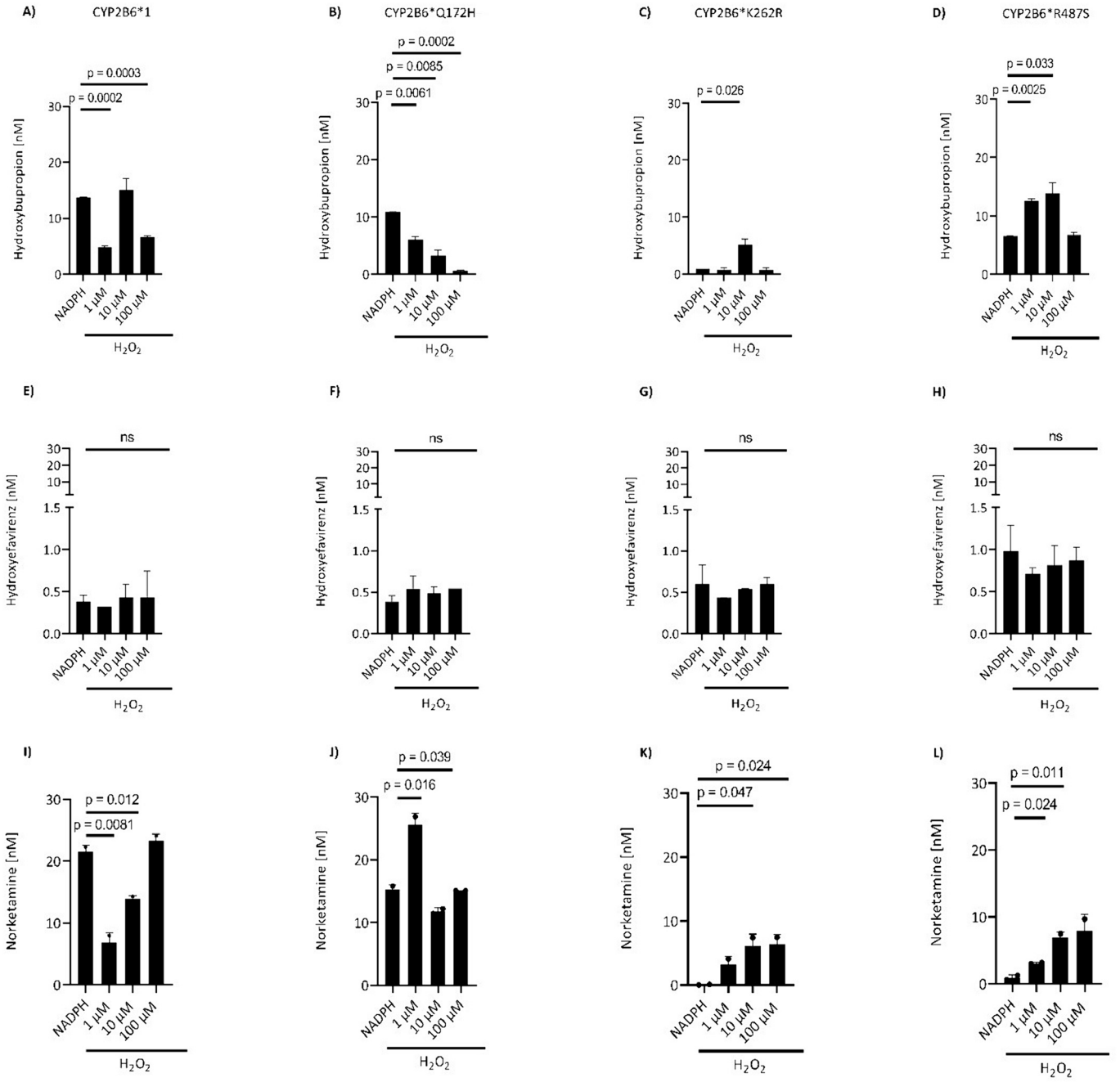

Fig. 5. 6-Hydroxybupropion (A-D), 8-hydroxyefavirenz (*E*-H) and norketamine (I-L) formation by CYP2B6*1, CYP2B6*Q172H, CYP2B6*K262R, CYP2B6*R487S after 1 h of incubation with either NADPH or 1 µM, 10 µM or 100 µM of H_2O_2. 10 µM of each substrate were incubated with each enzyme. Reaction was stopped and metabolite formation was determined by HPLC-MS/MS. First variance equality was verified by application of an F-test followed by an unpaired, two-tailed *t*-test with $n = 2$. All statistical analyses were performed with GraphPad Prism 9.4.1.

0.07 nM NK formation with NADPH.

Similarly, CYP2B6*R487S showed almost doubled HB formation with 10 µM H_2O_2 (14 ± 2 nM) compared to NADPH (7.0 ± 0.8 nM), and increasing NK formation at increasing H_2O_2 concentrations, with 100 µM resulting in the highest NK concentration (8.0 ± 2.4 nM).

In contrast to CYP2B6*K262R and CYP2B6*R487S, the variant CYP2B6*Q172H tended to show lower metabolite formation rates with H_2O_2 as the reaction driver. Weak metabolite formation was seen at low H_2O_2 concentrations (only a clear favouritism of H_2O_2 in ketamine metabolism when 1 µM H_2O_2 is added), while increasing H_2O_2 concentration decreased the NK formation. Bupropion metabolism was fully decreased by H_2O_2 in a concentration dependent manner.

Overall, the results indicate that both, ketamine and bupropion metabolism by the enzyme variants CYP2B6*K262R and CYP2B6*R487S are mostly mediated through the peroxide shunt pathway with H_2O_2 as a reaction driver. Furthermore, the variants differ in the preferred H_2O_2 concentration, since CYP2B6*K262R and CYP2B6*R487S show most bupropion metabolism at 10 µM H_2O_2, ketamine metabolism was most effective at higher concentrations of 100 µM H_2O_2. The third variant, CYP2B6*Q172H however showed higher ketamine turnover at the lowest H_2O_2 concentration of 1 µM and decreased turnover of bupropion and efavirenz when H_2O_2 is added as the reaction driver.

Hence, we observed a substrate dependent preference of the shunt

pathway and a variant dependent difference in H_2O_2 tolerance.

3.4. Role of recombinant CYP2B6*6 and CYP2B6*34 enzyme variants in H_2O_2 production

The third enzymatic pathway, possibly affecting the metabolism of bupropion, efavirenz and ketamine by CYP2B6 wild-type and variant enzymes is the uncoupling pathway. Uncoupling occurs independently of the peroxide shunt pathway and results in H_2O_2 production through the CYP450 reaction cycle. The uncoupling potential was studied by quantification of H_2O_2 production of recombinant CYP2B6*1, CYP2B6*6 and CYP2B6*34.

The enzymes were assayed without any substrate addition (Fig. 6A) and after 1 h incubation at 37 °C with 10 μM bupropion or efavirenz or ketamine (Fig. 6B-6D).

While all three enzyme variants showed basal H_2O_2 production, a significantly higher production was observed in CYP2B6*6 (25 ± 2 μM H_2O_2) and in CYP2B6*34 (64 ± 8 μM H_2O_2) compared to CYP2B6*1 (21 ± 3 μM H_2O_2) (Fig. 6A).

Addition of the three substrates in the medium did not affect peroxide production in CYP2B6*1 enzyme (Fig. 6B).

The CYP2B6*6 variant however was affected significantly by substrate addition with reduced H_2O_2 production (18.0 ± 2 μM H_2O_2) after addition of bupropion ($p < 0.0001$). Similarly, CYP2B6*34 peroxide production decreased to 50 ± 5 μM ($p = 0.08$) after addition of bupropion. Ketamine addition showed a significant decrease to 18 ± 3 μM ($p = 0.0011$) in CYP2B6*6 reaction, while CYP2B6*34 peroxide production remained unaffected by ketamine addition. Incubation with efavirenz resulted in no decrease of hydrogen peroxide in both variant enzymes. It is further notable that with bupropion and ketamine, only the substrates that were preferably metabolized *via* the shunt pathways in the single amino acid substituted CYP2B6 were able to decrease H_2O_2 production. This further supports the hypothesis that H_2O_2 is used as driver for the metabolism of ketamine and bupropion by CYP2B6*6 and CYP2B6*34.

3.5. Substrate influence on H_2O_2 production

Since the presented results so far indicate at a substrate dependent use of the shunt pathway in CYP2B6 and its variants, the substrate dependency of the coupling efficiency was studied. Therefore the enzymes were incubated with increasing (1 μM – 50 μM) bupropion, efavirenz and ketamine concentrations and the produced H_2O_2 was measured (Table 2).

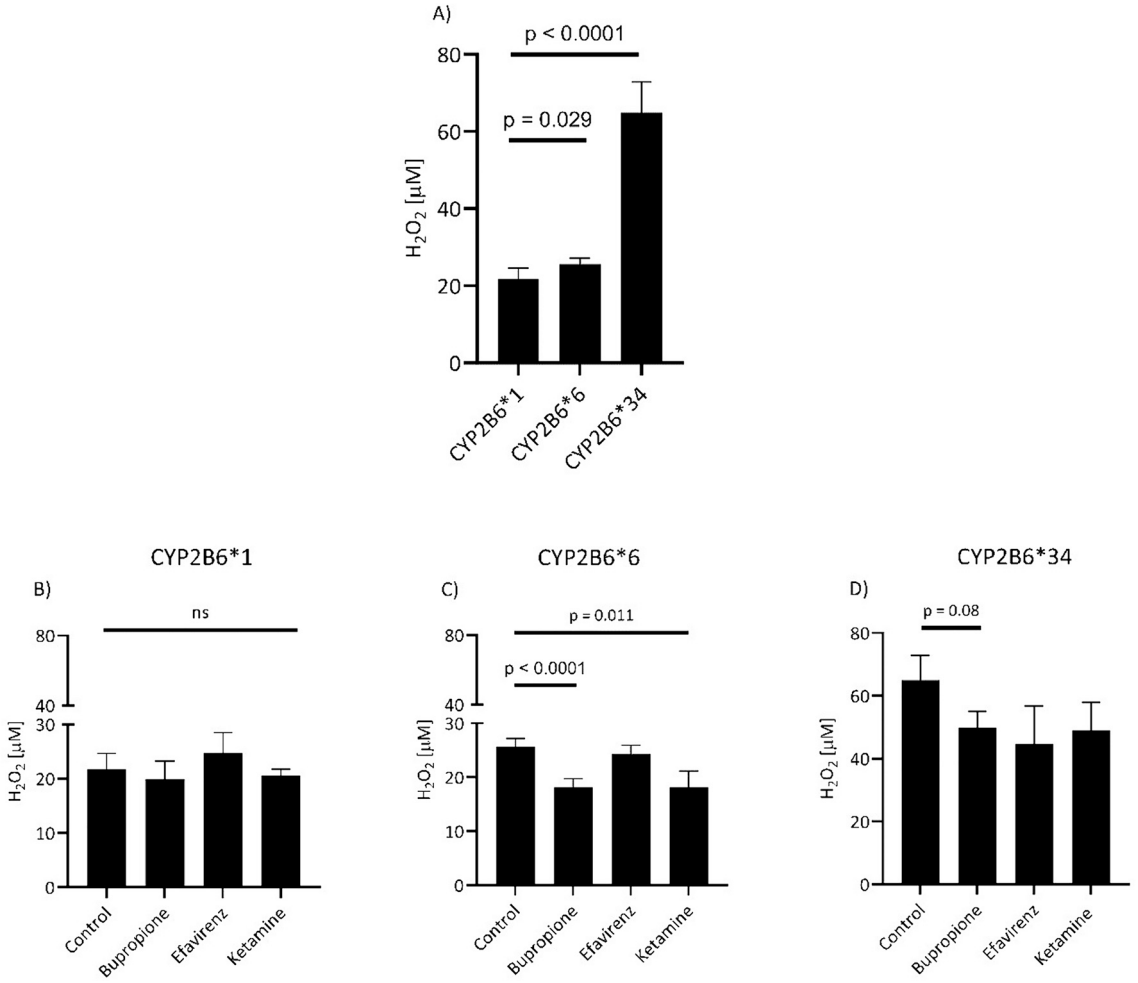

Fig. 6. H_2O_2 production by CYP2B6*1, CYP2B6*6 and CYP2B6*34 with and without the addition of substrates. Recombinant CYP2B6 enzymes were incubated for 1 h at 37 °C and H_2O_2 levels were fluorescently determined using AmplexRed as a probe. A) Determination of basic H_2O_2 levels in substrate absence. H_2O_2 levels after addition of 10 μM bupropion, efavirenz and ketamine are shown in B) - D). B) H_2O_2 levels of bupropion, efavirenz and ketamine metabolism by CYP2B6*1. ns = not significant. C) H_2O_2 levels of bupropion, efavirenz and ketamine metabolism by CYP2B6*6. D) H_2O_2 levels of bupropion, efavirenz and ketamine metabolism by CYP2B6*34. All data are presented as mean + SD and were calculated from three independent experiments. First variance equality was verified by application of a F-test followed by an unpaired, two-tailed *t*-test. All statistical analyses were performed with GraphPad Prism 9.4.1. Mean and standard deviation values are listed in the table.

Table 2
H_2O_2 production of CYP2B6*1, CYP2B6*6 and CYP2B6*34 enzymes with increasing concentrations of bupropion, efavirenz and ketamine. Recombinant CYP2B6 enzymes were incubated for 1 h at 37 °C with 1 μM, 10 μM and 50 μM bupropion, efavirenz and ketamine. H_2O_2 levels were fluorescently determined using AmplexRed as a probe. All data are presented as mean + SD and were calculated from two independent experiments. Group comparison was performed *via* an ordinary one way ANOVA. All statistical analyses were performed with GraphPad Prism 9.4.1. Significant results are indicate by the respective *p*-value in bold.

	Bupropion			**Efavirenz**			**Ketamine**		
	1 μM [μM ± SD]	10 μM [μM ± SD]	50 μM [μM ± SD]	1 μM [μM ± SD]	10 μM [μM ± SD]	50 μM [μM ± SD]	1 μM [μM ± SD]	10 μM [μM ± SD]	50 μM [μM ± SD]
CYP2B6*1	22.65 ± 2.98	30.15 ± 4.72	31.10 ± 2.15	22.93 ± 0.64	26.33 ± 3.01	30.010 ± 2.57	**21.40 ± 1.10**	**26.1 ± 0.5 $p = 0.018$**	**28.5 ± 1.70**
CYP2B6*6	24.34 ± 1.54	28.45 ± 1.45	32.26 ± 3.59	**25.13 ± 0.96**	**29.53 ± 2.25 $p = 0.007$**	**66.63 ± 8.78**	**47.22 ± 2.30**	**54.80 ± 1.88 $p = 0.025$**	**56.18 ± 0.23**
CYP2B6*34	**61.19 ± 3.57**	**74.59 ± 4.06 p = 0.0006**	**14.92 ± 0.5**	**44.63 ± 2.73**	**59.58 ± 3.41 p = 0.0162**	**73.55 ± 6.18**	**41.49 ± 0.21**	**56.88 ± 1.26 p = 0.0256**	**61.05 ± 4.17**

Again, CYP2B6*1 enzyme produced the least and CYP2B6*34 the highest amount of H_2O_2 compared to the variants. Concerning the effect of increasing substrate concentrations, ketamine metabolism lead to an increased H_2O_2 formation in all enzymes. In CYP2B6*1 an increase from 21 ± 1 μM with 1 μM ketamine to 26 ± 0.5 μM with 10 μM ketamine and 29 ± 2 μM with 50 μM ketamine was measured. This increase was determined to be significant with $p = 0.018$.

Increase of ketamine concentration also increased the H_2O_2 production of CYP2B6*6 and CYP2B6*34 significantly from 47 ± 2 μM to 56 ± 0.2 μM ($p = 0.025$) in CYP2B6*6 and from 41 ± 0.2 μM to 61 ± 4 μM ($p = 0.0256$) in CYP2B6*34. Ketamine is therefore a risk factor for increased uncoupling susceptibility irrespective of the genotype. Apart from ketamine, no other substrate altered the H_2O_2 production of CYP2B6*1 significantly.

Efavirenz concentrations increased the H_2O_2 production in CYP2B6*6 and CYP2B6*34. While CYP2B6*34 produced higher and increasing H_2O_2 concentrations at all three efavirenz concentrations (1 μM: 44 μM ± 3 μM H_2O_2; 10 μM: 59 μM ± 3 μM H2O2; 50 μM: 74 μM ± 6 μM, $p = 0.0162$), the increase in H_2O_2 from 10 μM efavirenz (29 μM ± 2 μM) to 50 μM efavirenz (67 μM ± 9) in CYP2B6*6 is the most significant with $p = 0.007$). Efavirenz is therefore more likely to increase the uncoupling susceptibility of CYP2B6*6 than CYP2B6*1 or CYP2B6*34.

In contrast, bupropion did not result in increased concentrations of H_2O_2 at the applied substrate concentrations in any of the three enzyme variants. CYP2B6*34 however seems to drop in H_2O_2 production from 75 ± 4 μM H_2O_2 with 10 μM bupropion to 15 ± 0.5 μM with 40 μM bupropion ($p = 0.0006$). This highly significant decrease can either be due to an increased metabolism of bupropion *via* the shunt pathway, thereby using up the produced H_2O_2 or by an early deactivation of the enzyme due to an H_2O_2 concentration exceeding the tolerated concentration.

Overall, especially the variant enzymes CYP2B6*6 and CYP2B6*34 were prone to reaction uncoupling with increasing substrate concentrations, allowing the conclusions, that this effect is substrate specific and reaction dependent.

4. Discussion

We investigated the functional effects of the exonic variants in the enzyme CYP2B6 determining the alleles *CYP2B6*6* and *CYP2B6*34*. For the *CYP2B6*6* allele, lower metabolism of efavirenz, and bupropion, and higher substrate metabolism of cyclophosphamide have been described, showing the multifaceted effects of the genetic polymorphisms encoded in this variant. While *CYP2B6*6* has been studied a lot in clinical and pharmacokinetic studies, *CYP2B6*34* and the single amino acid substitution R487S remain understudied. In order to understand the differential results in substrate metabolism, we studied the exonic amino acid variants occurring in *CYP2B6*6 and CYP2B6*34* in combination and separately. To uncover the uncoupling potential and catalytic abilities of recombinant CYP2B6*1, CYP2B6*6 and CYP2B6*34, the formation of hydrogen peroxide during the metabolism of bupropion, efavirenz and ketamine was assessed. The rationale behind the choice of substrates is based on the substrate specificity of CYP2B6 reactions that has already been observed with bupropion efavirenz and cyclophosphamide as detailed in the introduction. Due to the toxic nature of cyclophosphamide and the relatively new clinical application of ketamine, ketamine was chosen as a third substrate within this study.

With the aim to unveil the reason for our observed increased metabolism at lower substrate affinities of the enzyme variant, the respective single amino acid substitutions were investigated. While CYP2B6*K262R showed increased efavirenz metabolism compared to the Q172H substituted and the wild-type enzyme, bupropion and ketamine metabolism was significantly increased in the Q172H substituted CYP2B6 compared to CYP2B6*K262R. This effect has been described already in CYP2B6 metabolism of cyclophosphamide and efavirenz by [1]. The group studied the effects of the CYP2B6 amino acid substitution K262R alone on the metabolism of cyclophosphamide and efavirenz and compared it to the combined substitution of K262R and Q172H, hence the CYP2B6*6 phenotype.

Based on their data, the K262R mutant showed 142% catalytic efficiency of efavirenz metabolism compared to the wild-type enzyme. Addition of Q172H results in CYP2B6*6 enzyme and reduces the catalytic efficiency to 50%. In contrast, cyclophosphamide metabolism was decreased to 72% in CYP2B6*K262R and increased in CYP2B6*6 enzyme. The group therefore postulated that the Q172H substitution overcomes the effect of the K262R substitution. Whether Q172H addition increases or decreases the substrate metabolism is substrate dependent [1].

Similarly, our result showed that the CYP2B6*6 phenotype of all three substrates follow the phenotype observed in the single amino acid substitution Q172H. We therefore confirm the assumption that Q172H mainly accounts for the *CYP2B6*6* variant phenotype in efavirenz metabolism and add the metabolism of bupropion and ketamine to the affected substrates.

Besides CYP2B6*6, CYP2B6*34 enzyme activity was assessed as the combination of Q172H, K262R and R487S amino acid substitutions as well as in R487S amino acid substitution alone, since this is the only functionally differing enzyme alteration compared to CYP2B6*6. Significant differences between CYP2B6*6 and CYP2B6*34 enzymes with regard to activity were observed in bupropion and efavirenz metabolism. While bupropion metabolism was similar to the wild-type, efavirenz metabolism was highly increased in the CYP2B6*34 enzyme. Looking at the single amino acid substitutions, expression of R487S alone showed indeed increased efavirenz metabolism and decreased bupropion and ketamine metabolism activity compared to the Q172H. In addition, a significant increase in ketamine and bupropion metabolism was detected when comparing the R487S variant to the K262R variant. Radloff et al. too showed increased affinity of the R487S substitution towards bupropion and efavirenz and therefore hypothesized a wild-type like enzyme activity of the CYP2B6*34 enzyme based on its

combination with amino acid exchanges causing increased and decreased metabolic activity towards these substrates. We confirmed this assumption and showed that the CYP2B6*34 variant indeed has metabolic activity for bupropion, efavirenz that is similar to CYP2B6*1.

Therefore, R487S is the phenotype-determining substitution in the CYP2B6*34 mediated metabolism of bupropion, efavirenz and ketamine.

Since the inconsistencies in substrate binding affinity and enzymatic activity cannot be explained by an additive effect of the single amino acids, we systematically investigated functional differences that can be due to alternative enzyme reaction pathways in each of the variants. The novel findings of these experiments were twofold:

First, the use of H_2O_2 for activation of the shunt pathway is increased in bupropion and ketamine metabolism by all three variants CYP2B6*R487S, K262R and Q172H. Second, amino acid substitutions at presumably inactive protein-sites contribute to increased uncoupling susceptibility and hence H_2O_2 formation of CYP2B6 variants in a substrate dependent manner.

To investigate a possible link between the use of the shunt pathway and the increased metabolite formation of the variants, the single amino acid substitutions were expressed and turnover of bupropion, efavirenz and ketamine was determined with NADPH or H_2O_2 as the reaction driver.

Increased substrate oxidation in H_2O_2 supplied reactions compared to NADPH was considered as a use of the shunt pathway by the respective enzyme variant.

We only observed a favoured use of the shunt pathway in the metabolism of bupropion and ketamine, hence efavirenz metabolism seems to be constant in all variant as well as wild-type CYP2B6 enzymes (Fig. 5).

Interestingly, K262R is the only variant that exclusively showed enzyme activity with H_2O_2 as reaction driver in bupropion and ketamine metabolism. Considering that this variant showed the lowest metabolite formation before (Fig. 4), we assume that this amino acid substitution leads to an exclusive use of the peroxide shunt in bupropion and ketamine metabolism. Similarly, R487S substitution led to an increased metabolism of bupropion and ketamine with increased H_2O_2 concentrations (Fig. 5), indicating that this variant is capable of metabolizing these substrates through both productive reaction pathways.

The amino acid substitution Q172H showed a preferred use of the peroxide shunt at low H_2O_2 concentrations only in the metabolism of ketamine, while bupropion metabolism was highest with NADPH as a reaction driver and decreased with increasing H_2O_2 concentrations.

Taken together, we were able to observe divergent reaction pathway usage of the single amino acid substitution Q172H compared to K262R and R487S in the metabolism of bupropion. The use of the peroxide shunt as a productive reaction pathway was uniformly increased in the metabolism of ketamine with even the wild-type showing metabolic activity at highest H_2O_2 concentrations, indicating that this substrate induced the shunt pathway in CYP2B6 enzyme and enzyme variants. A further trigger for the use of the shunt pathway seems to be the K262R amino acid substitution, while this again must have a substrate specific character, since efavirenz metabolism did not increase with H_2O_2 as the reaction driver.

Considering that the combination of the amino acid substitutions in CYP2B6*6 and CYP2B6*34 showed decreased H_2O_2 levels in presence of bupropion and ketamine (Fig. 6), we can assume that this effect is due to the metabolism of the substrates through the shunt pathway, thereby consuming the produced H_2O_2. Based on this conclusion, we can further assume that the described effect of the single amino acids K262R and R487S on the use of the peroxide shunt is also present in the combined variants.

Under normal conditions, the two side reactions of peroxide shunt and reaction uncoupling are beneficial and contribute to the cellular redox homeostasis [45]. An aberrantly high use of the peroxide shunt however can lead to heme degradation and enzyme malfunction, thereby causing an increase in reaction uncoupling [36] leading to an abundance of H_2O_2 and accumulated reactive intermediates that cause an imbalance of cellular ROS.

The resulting oxidative stress can deleteriously affect local tissue integrity [35], cause oxidative DNA damage [43], membrane lipid peroxidation [20] and may contribute to pathogenesis of diseases as cancers, necrosis or chronic inflammation thereby increasing the patient's vulnerability to treatment-related side effects. While to date it is still difficult to assess the extent of CYP450 enzyme contribution to the *in vivo* oxidative stress, *in vitro* experiments unambiguously showed the CYP450 uncoupling and hence ROS production capacity. In that sense, mammalian CYP450 can show coupling efficiencies as low as 1.2% as for CYP1A2 mediated 7-ethoxycoumarin metabolism [32] and as high as 97% for CYP17A1 mediated pregnenolone metabolism [23]. This wide range in coupling efficiency is explainable by the high substrate promiscuity of CYP450 enzymes. When only looking at drug metabolizing CYP450, highest coupling efficiencies are between 40% and 50% at maximum [6,11], with most enzyme-substrate couples showing coupling efficiencies around 5% - 20% [10,16,19,33]. Accordingly, the substrate structure influences the enzymatic coupling efficiency. Furthermore, genetic alterations and structural differences may account for an increased uncoupling. Both possibilities were studied in the presented study.

Due to the *in vitro* character of the study, enzymatic stability needs to be considered when interpreting the given data. Human CYP450 enzymes have been known to show fast deactivation and heme degradation. Therefore in the presented study, agents that were determined to be stabilizing for CYP450 enzymes have been added to the reaction buffer, partial enzymatic inactivation cannot be excluded. This can be a further uncoupling trigger and may even be causally related to the genetic variances. To minimize this uncertainty, the integrity of the expressed enzymes was spectrophotometrically assessed through CO-binding assays (Fig. 1) and the incubation time was reduced from 24 h to 1 h.

Indeed, significantly increased H_2O_2 production by recombinant expressed human CYP2B6*6 and CYP2B6*34 was detected which may indicate an increased risk for ROS production. Especially the increase in CYP2B6*34 exceeded CYP2B6*6 by 156% and the wild-type enzyme by 205%, while CYP2B6*6 only showed a slight increase of 19% compared to the wild-type enzyme. Since these two variants only differ by the R487S substitution, we assume that the R487S substitution is primarily responsible for the increased uncoupling susceptibility of this variant. While no studies have linked this specific amino acid substitution to increased uncoupling yet, we and others have shown in a previous study that genetic variants in other CYPs such as CYP2C9*3 [46], CYP2C8*3 [50] and even CYP2B6*4 [6] are prone to enzyme uncoupling, and thus may act as risk factors for increased ROS production.

We furthermore observed an increase in H_2O_2 production by CYP2B6*6 and - to a greater extent - by CYP2B6*34 with addition of increasing substrate concentrations of ketamine and efavirenz. We therefore conclude a causal correlation between the substrate and the increased ROS formation of CYP2B6*6 and CYP2B6*34. Especially ketamine was able to increase the uncoupling of all enzyme variants, including the wild-type and is therefore considered, to be a general uncoupler for CYP2B6. The applied concentrations showed that a significant increase of ketamine-induced uncoupling starts at 1 μM (0.237 μg/ml) in the variant and 10 μM (2.37 μg/ml) in the wildtype enzyme. Based on the *Pfizer* professional information of *Katenest®S*, a plasma peak concentration of 0.14 μg/ml (corresponding to 0.6 μM) is reached after 25 min post intramuscular injection with a bioavailability of 93%. Other application forms of ketamine such as intravenous injection for general anaesthesia can even yield plasma C_{max} values of up to 5–10 μM [51]. We can therefore assume that this effect could be of physiological relevance, especially in *CYP2B6*6* and *CYP2B6*34* carriers, making these patients vulnerable to inflammatory load by ROS formation under ketamine treatment.

In the presented study, only three CYP2B6 substrates have been

assessed, already showing a substrate specific effects, which stresses the importance of a systematic determination using all endogenous and exogenous substrates.

Furthermore, this observed effect of substrate specific uncoupling has been discussed before in the scope of drug induced toxicity [42], while systematic analyses of specific substrate – enzyme couples have so far only been performed in zebrafish CYP1A2 [14] and CYP450-cam [3,28]. All three studies identified substrate structure and CYP isoforms with amino acid exchanges at the substrate binding region as triggers for reaction uncoupling.

Overall, our results suggest that reaction uncoupling and substrate metabolism through the peroxide shunt are both increased in CYP2B6 variants encoding the K262R substitution. We therefore postulate that an increased use of the peroxide shunt on the one hand leads to increased substrate turnover but also to enzyme malfunction and heme oxidation leading to increased uncoupling and hence oxidative imbalance. This increases the individual's inflammatory response leading to pathological phenotypes as cancers or necrosis. This phenomenon is apparently further dependent on the substrate and in our case, ketamine seems to be a risk factor for this negative loop in enzymatic reaction. Since the causative mutations can apparently also be located in inactive protein sites, a model based prediction of these effects is impossible and laboratory experimental investigations are needed.

This shows that pharmacogenetic effects are not limited to drug clearance alone but also may affect enzyme function, and this in turn can affect ROS formation and inflammatory symptoms and diseases. These functional pharmacogenetic effects cannot be compensated by dosing adjustments alone. Additionally, our knowledge on pharmacogenetic variability and the effect of variants on enzyme function is rather small and not yet covering the genome of most African ethnicities, even if the pangenome shows that the African continent belongs to the most diverse yet understudied populations. More knowledge on genetic variation in CYP450 drug metabolizing isoforms and its functional role would potentially allow future predictions and prevention of this toxic side reaction, increasing the possibilities for patient and population specific drug treatment.

CRediT authorship contribution statement

Sabrina Yamoune: Writing – original draft, Visualization, Project administration, Investigation, Data curation, Conceptualization. **Julian Peter Müller:** Writing – review & editing, Validation, Formal analysis. **Immaculate Mbongo Langmia:** Writing – review & editing, Project administration. **Catharina Scholl:** Writing – review & editing, Supervision, Resources. **Julia Carolin Stingl:** Writing – review & editing, Validation, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

None of the authors declared any conflict of interest with the work that has been published in this article.

Data availability

Data will be made available on request.

Acknowledgments

Funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) – Project number 455749974.

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