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Recombinant Production of Human Microsomal Cytochrome P450 2D6 in the Methylotrophic Yeast *Pichia pastoris*

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Microsomal cytochrome P450 monooxygenases of groups 1–3 are mainly expressed in the liver and play a crucial role in phase 1 reactions of xenobiotic metabolism. The cDNAs encoding human CYP2D6 and human NADPH-P450 oxidoreductase (CPR) were transformed into the methylotrophic yeast Pichia pastoris *and expressed with control of the methanol-inducible AOX1 promoter. The determined molecular weights of the recombinant CYP2D6 and CPR closely matched the calculated values of 55.8 and 76.6 kDa. CPR activity was detected by conversion of cytochrome c by using isolated microsomes. Nearly all of the recombinant CYP was composed of the active holoenzyme, as confirmed by reduced CO difference spectra, which showed a single peak at 450 nm. Only by coexpression of human CPR and CYP was CYP2D6 activity obtained. Microsomes containing human CPR and CYP2D6 converted different substrates, such as 3-cyano-7-ethoxycoumarin, parathion and dextrometorphan. The kinetic parameters of dextrometorphan conversion closely matched those of CYP2D6 from other recombinant expression systems and human microsomes. The endogenous NADPH-P450 oxidoreductase of* Pichia pastoris *seems to be incompatible with human CYP2D6, as expression of CYP2D6 without human CPR did not result in any CYP activity. These recombinant strains provide a novel, easy-to-handle and cheap source for the biochemical characterisation of single microsomal cytochromes, as well as their allelic variants.*

Introduction

Mammalian cytochrome P450 monooxygenases (CYP; E.C. 1.14.14.1) are ubiquitous proteins that are involved in the oxidation of several endogenous compounds, such as steroids, prostaglandins and fatty acids. Group 1, 2 and 3 CYPs also play a key role in the metabolism of xenobiotics, which consists of two phases. In phase 1, called *functionalisation,* CYPs are responsible for the addition of functional groups to foreign compounds by hydroxylation, dealkylation, deamination etc. In phase 2, known as *conjugation,* transferases use those groups to couple charged molecules or groups making the modified compound more water soluble, which allow their efficient excretion.

Although the CYPs are expressed at different levels in many tissues, the liver contains 90% of those CYPs relevant for detoxification. In contrast to procaryotic CYPs, the respective eucaryotic proteins are membrane bound and are mainly found on the cytosolic side of the endoplasmatic reticulum. The activity of the CYPs depends greatly on electron transfer from NADPH to the heme group of the CYPs by accessory proteins like NADPH cytochrome P450 reductase (CPR; EC 1.6.2.4) and cytochrome b5 (Cyb5).

Broad substrate specificities, with individually different phenotypes due to polymorphisms, duplications and different expression levels are characteristic of those microsomal CYPs. Thus, they play a crucial role in drug interactions and are therefore highly important to the pharmaceutical industry. Within the last 15 years, in vivo experiments on animal models have been replaced by in vitro studies on human enzymes from different sources.[1–6] Microsomal preparations or cellular systems, such as liver slices and human hepatocytes, are used and these provide the advantage of a maintained cellular integrity in relation to other enzymes, cofactors, transporters etc., contributing to the activity, as well. Whereas all those systems contain a complete set of enzymes, recombinant expression systems allow the production of single enzymes for specific applications, such as drug metabolism. In contrast to the human sources, the availability of recombinant enzymes is not limited. Currently, several recombinant expression systems are under investigation: mammalian systems that provide transient (COS, HepG2) and permanent (V79) expression of individual human CYPs are complicated and expensive to handle and often exhibit low levels of functional enzyme.[4,7,8] However, some of them are commercially available. Among the simpler

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systems, mainly baculovirus-infected insect cells are currently used to purify microsomes containing individual mammalian CYPs and accessory proteins (CPR and Cyb5) at reasonable levels.[9] Drawbacks of the insect-cell-based systems are the complicated cultivation of the insect cells and the expensive media containing heme or heme precursors, which make scaling-up difficult. Due to the cheap cultivation procedures and media required and ease of handling, *E. coli* was also used as the host for the expression of a couple of CYPs.[10–12] However, a truncation or at least modification of the N-terminal membrane anchor sequence was required.[13] For the expression of some, but not all CYPs in *E. coli,* the addition of heme or its precursors is needed. The yeast *Saccharomyces cerevisiae* combines the ease of handling of prokaryotic systems with the features of eukaryotic systems, such as post-translational processing. Whereas initial experiments did not provide reasonable cytochrome P450 expression levels, Pompon's group was able to improve the *S. cerevisiae* system substantially by coexpression of Cyb5, CPR,[14,15] and, later, also potentially required phase 2 enzymes, such as epoxide hydrolase.[5,16] Meanwhile, a couple of mammalian microsomal CYPs have been expressed in *S. cerevisiae*.[17–19] Moreover, some unconventional yeasts, such as *Schizosaccharomyces pombe* or *Yarrowia lipolytica,* have been used for the expression of human cytochromes P450.[20,21]

Due to the high expression yields of soluble proteins obtained by intracellular and secreted expression and ease of handling, in recent years the methylotrophic yeast *Pichia pastoris* has gained in popularity as an expression system. A couple of proteins have been expressed in very high yields (up to 10 gL^{-1}).[22,23] Despite additional advantages of *Pichia* over the common baker's yeast, such as a glycosilation pattern that is closer to the human one than that of *S. cerevisiae*, only three eukaryotic CYPs from spiny dogfish shark, spiny lobster and cassava have been expressed so far;[24–26] none of them is of human origin.

Here, we present the establishment of *Pichia pastoris* as an expression system for the production and characterisation of human microsomal CYPs. CYP2D6, the model enzyme that we chose, is a polymorphically expressed microsomal cytochrome P450,[27] which almost exclusively catalyses the conversion of more than 50 relevant drugs, including cardiovascular drugs, β-adrenergic blocking agents, tricyclic antidepressants and opioid derivatives.[28,29] Overall, at least 72 different CYP2D6 alleles exist: splice variants, frame-shift mutations, deletions and premature stop codons result in a complete defect of the enzyme. 5–10% of the Caucasian population carry this phenotype.[30] Other genotypes show significant phenotype alterations due to shifted kinetic properties or varied expression levels.[31] People lacking CYP2D6 activity accumulate metabolites that cannot be metabolised by other CYP varients. In order to allow the analysis of the relevant CYPs and their variants separately, a simple and fast recombinant expression system is needed.

Results

Cloning and Expression of CYP2D6 and CPR

The genes encoding CYP2D6 and CPR were cloned and ligated separately into the vector pPICZ A under control of the AOX1 promoter, as described in the Experimental Section. Upon linearisation, both vectors pPICZ-2D6 and pPICZ-CPR (Figure 1) were transformed into *Pichia pastoris* X-33, and chromosomal integration of the heterologous expression cassettes was confirmed by PCR with genomic DNA as the template and gene-specific primers (data not shown). Since expression levels in recombinant *Pichia* clones tend to show clonal variation, five transformants of each construct were investigated for heterologous protein expression: microsomes were isolated from samples taken 90 h after induction, and the protein content was determined. Aliquots containing ~20 μg microsomal protein were analysed by Western blotting. Bands of the correct size of ~50 kDa (CYP2D6) and ~77 kDa were detected in the lanes of all investigated samples. The band corresponding to CYP2D6 is shown in lane 1 of Figure 2 A and is exemplary for the CYP2D6-expressing clones (*P. pastoris* X33/pPICZ-2D6).

Reductase activity (conversion of cytochrome c) was detected in microsomal fractions of all clones harbouring the CPR gene (Figure 6, below). Nontransformed *Pichia* strains or strains transformed with pPICZ-2D6 or pPICZ A (both without a heterologous reductase gene) did not show any reductase activity, although *P. pastoris* contains its own endogenous oxidoreductase.

In order to determine CYP2D6 activity qualitatively, a modified Ellman's assay based on the inhibition of acetylcholine esterase by paraoxon (an insecticide) was established. Paraoxon is generated in a CYPD6-catalysed reaction by oxidation of par-

Figure 1. Map of the coexpression vector pPICZ-CPR-CYP2D6. 5′ AOX1: alcohol oxidase 1 promoter region allows methanol-inducible expression in *P. pastoris*, *Sh* ble: zeocin resistance gene derived from *Streptoalloteichus hindustanus*, AOX1 TT region: native transcription termination and polyadenylation signal from AOX1 gene permits mRNA processing, CPR: sequence encoding human oxidoreductase, CYP2D6: sequence encoding CYP2D6. The expression cassette of CYP2D6 is shown in grey, that of CPR in white. The origin of replication functional in *E. coli* and the transcription termination region from *S. cerevisiae* that allows processing of *Sh* ble mRNA are not marked the figure.

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Figure 2. Western blot analysis of microsomes taken 90 h after induction from cultures of *P. pastoris* X-33 transformed with pPICZ-CYP2D6 and pPICZ-CPR-CYP2D6, respectively. Proteins were detected with A) *anti*-CYP2D6- and B) *anti*-CPR-antibodies. Lane 1: clone 2D6-8*; lane 2: negative control X-33/pPICZ A; lanes 3–6: microsomes of coexpression clones C1–C4; lane 7: prestained protein standard (Invitrogen); lane 8: unstained protein standard (BioRad); lanes 9 and 10: commercial preparation of CYP2D6 and oxidoreductase recombinantly expressed in *E. coli* as positive controls (New England Biolabs): 1:10 dilution and undiluted, respectively.

athion. In parallel, a fluorescence assay based on the de-ethylation of 3-cyano-7-ethoxycoumarin was used for the direct determination of CYP2D6 activity. All clones expressing only CYP2D6 showed ~95% residual acetylcholine esterase activity. Thus, no significant CYP2D6 activity could be measured (Figure 3, 2D6-1, -2, -3 and -4). Moreover, de-ethylation of 3-cyano-7-ethoxycoumarin (Figure 4) was not obtained when using microsomes of these strains. Even a mixture of recombinant *Pichia* microsomes containing human CYP2D6 and human CPR did not result in any activity (Figures 3 and 4) towards either of the two substrates.

Human liver microsomes, as well as commercially available *E. coli* membrane fractions containing truncated CYP2D6 variants and human oxidoreductase, served as positive controls and showed activity with both assays (data not shown).

Because mixtures of microsomes containing properly folded CYP2D6 (as confirmed by CO difference spectrum, data not shown) and microsomes containing active CPR did not yield any CYP activity, the limiting factor seemed to be the electron transfer.

Coexpression of CYP2D6 and CPR

In order to improve the electron-transfer system, clones coexpressing human CPR and CYP2D6 were generated: the complete expression cassettes of both enzymes—each including promoter and terminator—were combined within one vector by using a cloning strategy that was recently used for the coexpression of heavy and light chains to produce functional, active Fab fragments.[32] After transformation of the resulting vector pPICZ-CPR-CYP2D6 into *P. pastoris* X33, the chromosomal integration of both expression cassettes was confirmed for all tested transformants by PCR by using CPR- and CYP2D6-specific primers. All tested clones contained both genes (Figure 5).

The microsomal expression of CYP2D6 and oxidoreductase, as well as the activity of the enzymes, was tested as described for the separate expression of both genes.

Protein bands corresponding to the correct size of CYP2D6 and CPR were detected by Western blot analysis in the microsomal fractions of all four investigated clones (Figure 2). Whereas a single strong band of ~50 kDa was detected by probing the blot with anti-CYP2D6 antibody (Figure 2 A, lanes 3–6) a strong band of ~77 kDa and a weaker one of ~58 kDa were detected when probing the blot with CPR-specific antibodies (Fig-

Figure 3. Ellman test for the detection of CYP2D6 activity by inhibition of recombinant acetylcholine esterase. Microsomes were isolated 90 h after induction. RA: acetylcholine esterase residual activity; C1–C4: microsomes of coexpression clones C1–C4; 2D6-1–2D6-4: microsomes of clones expressing CYP2D6; CPR-1 and CPR-2: microsomes of clones expressing CPR, pPICZ A: microsomes of *P. pastoris* X-33 harbouring pPICZ A (negative controls are grey), 2D6+CPR: mixtures of microsomes from clones containing pPICZ-CPR or pPICZ-CYP2D6.

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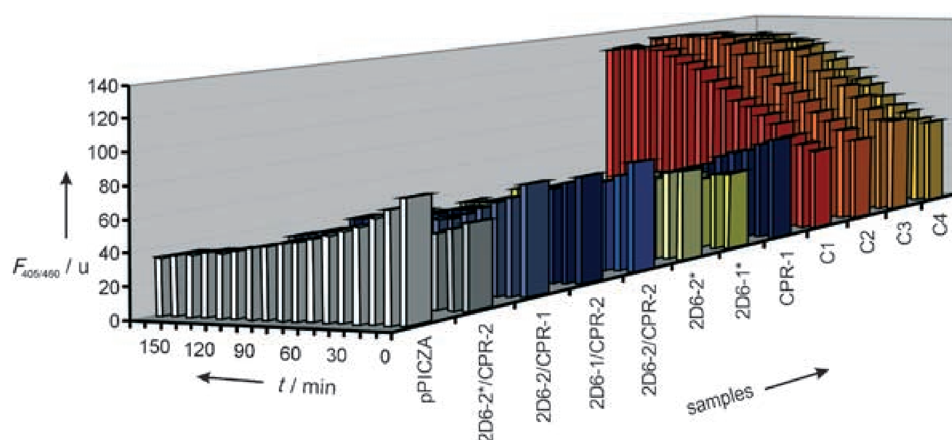

Figure 4. De-ethylation of 3-cyano-7-ethoxycoumarin was measured by using a fluorescence assay (excitation at 405 nm, emission at 460 nm). Microsomes of recombinant *Pichia* strains were isolated after 90 h of induction. F: fluorescence; C1–C4 microsomes of coexpression clones; 2D6-1 and 2D6-2 microsomes of clones expressing CYP2D6; CPR: microsomes of clones expressing CPR, pPICZ A microsomes of a clone transformed with pPICZ A (negative control), 2D6+CPR mixtures of microsomes of clones expressing CYP2D6 and CPR.

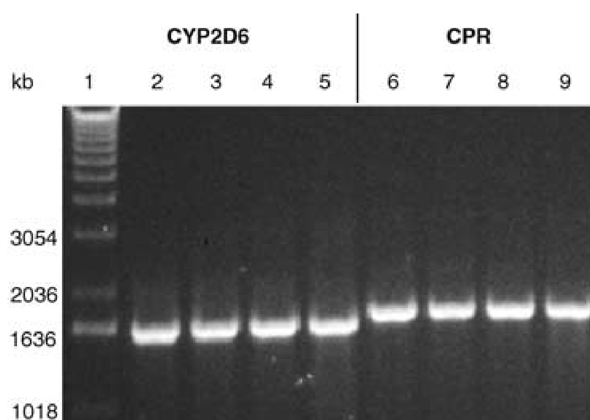

Figure 5. Confirmation of genomic integration of expression cassettes encoding CYP2D6 and CPR by PCR by using gene-specific primers. Lane 1: 1 kb ladder, lanes 2–5: PCR with genomic DNA of clones C1–C4 with primers specific for CYP2D6, lanes 6–9: PCR with genomic DNA of clones C1–C4 with primers specific for CPR.

ure 2B, lanes 3–6). All four clones coexpressing CYP2D6 and oxidoreductase showed strong CPR activity as shown in Figure 6 (lanes C1–C4).

When using the modified Ellman test, microsomes of the four investigated coexpression clones showed significant CYP2D6 activity: the residual acetylcholine esterase activities obtained were between 55 and 40% (Figure 3, C1–C4), in contrast to 95% obtained with samples from clones expressing CYP2D6 only (Figure 3, 2D6-1–2D6-4). As expected, no acetylcholine esterase inhibition was obtained with microsomes from clones expressing the oxidoreductase only and clones containing the vector pPICZ A (both serving as negative controls; Figure 3, CPR-1 and -2; pPICZ A). These results were confirmed by two assays based on the conversion of two typical substrates of CYP2D6, 3-cyano-7-ethoxycoumarin Figure 4 and dextrometorphan Figure 7. Only microsomes of the coexpression clones catalysed the de-ethylation of 3-cyano-7-ethoxycoumarin as shown in a fluorescent assay (Figure 4, C1–C4), whereas microsomes from all other clones, including those of the negative controls, those expressing CYP2D6 only and mixtures of those expressing CYP2D6 or CPR, did not lead to any substrate conversion. Similar results were obtained for the hydroxylation of dextrometorphan, as determined by HPLC (Figure 7). Only microsomes of clones expressing both CYP2D6 and CPR showed conversion of dextrometorphan (retention time ~19 min) to dextrorphan (DP, retention time ~8.7 min). Microsomes of clones expressing CYP2D6 only or negative controls did not show any product peak.

In order to quantify the CYP expression, reduced CO difference spectra, total protein concentrations and activities of the microsomal fractions from clones coexpressing CYP2D6 and CPR were measured. The CO difference spectrum in Figure 8 clearly shows a peak at 450 nm corresponding to the holo enzyme containing the heme group. As there is no peak at 420 nm, all the CYP protein seems to be correctly folded. The 450 nm peak revealed a CYP2D6 content in the microsomal fraction of ~2.7 nmol$\,$mL^{-1} or 0.12 nmol$\,$mg^{-1} total protein. The specific activity of the demethylation of dextrometorphan resulted in ~5 pmol DP per pmol CYP per min, as determined by LC/MS. The kinetic parameters concerning the conversion of dextrometophan (V_{max} = ~8.8 pmol$\,$min^{-1} per pmol enzyme, K_M ~1.9 μM, V_{max}/K_M ~2.5 μL per pmol enzyme per min) are in

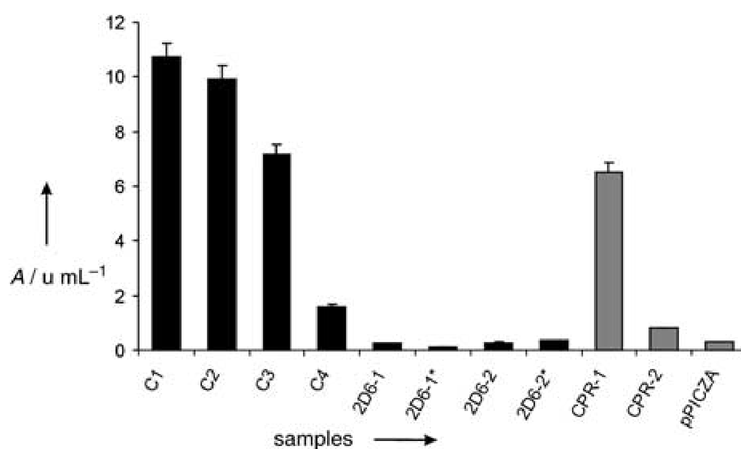

Figure 6. Oxidoreductase activity of recombinant human CPR was determined by conversion of cytochrome c. A: activity; C1–C4 microsomes of different coexpression clones (CYP2D6/CPR); 2D6-1–2D6-2 microsomes of different clones expressing CYP2D6; CPR microsomes of clones expressing CPR, 2D6+CPR mixtures of microsomes expressing CYP2D6 and CPR, pPICZ A microsomes of clones containing the empty vector (negative controls are grey).

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Figure 7. The demethylation of 5 μM dextrometorphan by CYP2D6 containing microsomes isolated from a *P. pastoris* X-33 harbouring pPICZ-CPR-CYP2D6 90 h after induction was measured by HPLC. The output of the fluorescence signal (FS) is in volts due to a potential transformer integrated into the system.

Figure 8. The CO difference spectrum of recombinant CYP2D6 clearly shows only one peak at 450 nm corresponding to the correctly folded holoenzyme.

the same range as those measured with CYP2D6 from other recombinant expression systems, as shown in Table 1.

Discussion

As already outlined in the Introduction, microsomal cytochromes P450 play a crucial role in the phase 1 reactions of xenobiotic metabolism. The human ones especially are needed by many pharmaceutical companies to test drug candidates for adverse drug interactions and thus are of great economic interest.

In this study, we have shown the coexpression of functional human CYP2D6 and CPR in the methylotrophic yeast *P. pastoris*. Strains coexpressing CPR and CYP2D6 each under the control of the methanol inducible AOX1 promoter were generated by a strategy previously used for the coexpression of heavy and light chains of a Fab fragment.[32]

Besides zeocin, there is no other suitable antibiotic as a selection marker for *P. pastoris* commonly in use. Several auxotrophy markers are used but, due to their low efficiency, the screening of a high number of colonies is generally required when using them. This, in turn, is not suitable when screening for microsomal CYPs, due to the elaborate preparation of microsomal fractions. Thus, a prerequisite was the generation of a coexpression vector containing both expression cassettes. All colonies screened after transformation with this coexpression vector integrated both expression cassettes into the genome, as shown by PCR. Those strains expressing both human CPR and CYP2D6 exhibited activity, as shown by the conversion of different typical CYP2D6 substrates (dextrometorphan, 3-cyano-7-ethoxycoumarin), but also uncommon substrates such as parathion.

The presented assay, based on the oxidation of parathion followed by the inhibition of the acetylcholine esterase, provides a fast and easy-to-handle photometric assay for the detection of CYP activity. It does not require any pretreatments, such as extractions, and there is no need for complicated and expensive devices such as HPLC, GC/MS or LC/MS. The substrate parathion is much cheaper than most of the fluorimetric substrates, such as coumarin derivatives. In addition to the detection of CYP activity, the assay can be used for monitoring phosphorothionate pesticides, as we have recently shown.[33]

A comparison of the kinetic parameters of CYP2D6 from different recombinant expression systems (COS7, Baculovirus, *E. coli*, yeast) is provided in Table 1. It shows that the enzyme we expressed in *P. pastoris* has similar properties in relation to the conversion of dextrometorphan. The expression level in *P. pastoris* (120 pmol CYP2D6 per mg protein) is in the same range as that obtained in Baculovirus-transformed insect cells and *E. coli*, but significantly higher than HepG2 and other mammalian systems. However, these comparisons lack significance, due to the different and imprecise methods (Western blotting, reduced CO difference spectra) used for the determination of microsomal CYPs in the different reports. When using the same protocol for the demethylation of dextromethorphan, microsomes from recombinant *P. pastoris* showed fivefold higher substrate conversion rates (4.8 pmol per min per pmol protein) than microsomes from Baculovirus-transformed insect cells purchased commercially from BD Gentest (1 pmol per min per pmol protein), as determined by LC/MS.

Expression of CYP2D6 without the coexpression of human CPR was also possible, as shown by Western blotting, but did not yield any activity. This was unexpected, as a couple of endogenous electron-transfer systems are encoded in the genome of *P. pastoris*: the endogenous genes encoding a NADPH-cytochrome P450 reductase, a NADH-cytochrome b5 reductase and a cytochrome b5, have to be especially men-

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Table 1. Concentration and kinetic parameters of CYP2D6 from *P. pastoris* compared with CYP2D6 from other expression systems and human liver microsomes.

Host system	Purification	Concentration [pmol per mg protein]	K_M [μM]	V_{max}[1] [pmol pmol^{-1} min^{-}]	V_{max}/KM [μL pmol^{-1} min^{-1}]	Ref.
P. pastoris	micros.	120	1.9	4.8	2.5	–
S. cerevisiae	micros.	51	n.d.	n.d.	n.d.	[19]
S. cerevisiae	micros.	12	n.d.	n.d.	n.d.	[18]
S. cerevisiae	micros.	250	8.5	10	1.2	[17]
E. coli	micros.	306	1.1	2.7	2.5	[42]
baculovirus	micros.	n.d.	1.0	2.4	2.4	[31]
baculovirus	pur. CYP	n.d.	1.9–3	8.5–9.0	n.d.	[43]
baculovirus	pur. CYP	n.d.	3.7	11.9	3.2	[44]
COS 7 cells	micros.	17–30 pmol per transfection	5.4	0.68	0.13	[31]
HepG2	micros.	35–45	n.d.	n.d.	n.d.	[45]
	lysate	15–25				
lymphoblasts		160	n.d.	n.d.	n.d.	[46]
human liver	micros.	n.d.	2.7	8	3	[42]
human liver	micros.	5	n.d.	n.d.	n.d.	[47]
human liver (38 samples)	micros.	8–115	n.d	n.d.	n.d.	[48]
human liver (16 samples)	micros.	n.d.	2.6–15	22–500 pmol mg^{-1} min^{-1}	n.d.	[49]

n.d.: values either not determined or not provided by the authors

tioned in this context. These results are in contrast to other studies, which reported that endogenous electron-transfer systems in other yeasts are fully compatible with human CYPs: Bureik and colleagues reported that the inner mitochondrial electron-transfer system of *S. pombe* sufficiently transferred electrons to recombinantly expressed CYP11B1.[20]

Even a mixture of *Pichia* microsomes containing recombinant human oxidoreductase with microsomes containing recombinant human CYP2D6 did not result in any activity. However, this is probably due to a lack of fusion of the microsomes and might be overcome by improvement of the protocol.

Various soluble proteins have been expressed, both secreted and not secreted in *P. pastoris,* in the grams per litre range. Although some CYPs from spiny lobster, spiny dogfish shark and cassava have been expressed in *P. pastoris* ,[24–26] this is the first report of a mammalian, microsomal CYP expressed in a methylotrophic yeast. Additionally, it is one of only few reports describing the coexpression of different proteins in one *P. pastoris* strain.[22,32,34]

The chosen cloning strategy allows the generation of clones expressing additional proteins. For example, coexpression of enzymes of the phase 2 reactions with respective CYPs are of relevance when simulating complete degradation pathways of certain xenobiotics, as occur in mammalian cells or tissues. However, simulating the in vivo system in hepatocytes requires the adjustment of the correct expression-level ratios of the respective enzymes. This can be achieved by coexpression of CYP and CPR under different controllable promoters or after single expression of the enzymes by mixing the desired amounts of microsomes as described above.

The yeast *Pichia pastoris* provides an easier-to-handle and much cheaper alternative to the mammalian or viral expression systems that are currently used, in most cases for the production of recombinant mammalian CYPs (see Table 1). Due to its simple handling, it allows the fast generation of allelic variants and genotypes of single microsomal cytochromes and their biochemical characterisation.

Furthermore, preliminary results show that recombinant *Pichia* coexpressing human CYP and CPR are suitable for biotransformations of hydrophobic, pharmacologically relevant substrates with whole cells, thus avoiding the isolation of microsomes, which is currently the limiting step (data nnot shown). Currently, this is done with recombinant *E. coli* but, as described above, only with N-terminally modified CYP variants.[11,12] Thus, such biotransformations with *Pichia* strains coexpressing phase 1 and 2 enzymes would raise the possibility of a biotechnological production of metabolites on a larger scale and might serve as an alternative to chemical synthesis.

The high substrate conversion rates combined with ease of handling, including transformation, cultivation and scaling-up make *Pichia* superior to other systems, such as CHO-cells, hepatocytes, baculovirus-transformed insect cells, but also yeast systems like *S. pombe* and *S. cerevisiae*.

Experimental Section

Chemicals: Unless stated otherwise, all chemicals were purchased from Sigma–Aldrich Chemie (Steinheim, Germany), Fluka (Buchs, Germany), Merck (Darmstadt, Germany) or Riedel de Haen (Seelze, Germany), at the highest purity available. 3-Cyano-7-ethoxycoumarin and 3-cyano-7-hydroxycoumarin were obtained from Molecular Probes (Mo Bi Tec, Göttingen, Germany). Oligonucleotides were purchased from Sigma ARK GmbH (Darmstadt, Germany).

Microorgansims, plasmids and growth conditions: *E. coli* DH5α [F^{-} *end*A1 *hsd*R17(rk^{-}, mk^{+}) *sup*E44 *thi*-1 λ^{-} *gyr*A96 *rel*A1 Δ(*arg*F-*lac*zya)U169] was used for the cloning steps and propagation of all

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expression vectors. Cells were cultivated in $LB_{low\,salt}$ (5 g L^{-1} yeast extract, 10 g L^{-1} peptone, 5 g L^{-1} NaCl) supplemented, if required, with zeocin (25 mg L^{-1}; Duchefa Biochemie B.V., Haarlem, The Netherlands) at 37 °C and 200 rpm.

Pichia pastoris X-33 (Invitrogen) was used for the expression of recombinant CYP2D6 and CPR. The following media were employed in the cultivation of *Pichia* cells under different conditions: YPD medium (yeast extract (1%), peptone (2%) and glucose (2%)); YPDS medium (YPD medium supplemented with sorbitol (1 M)); BMGY medium (yeast extract (1%)), peptone (2%), potassium phosphate buffer (100 mM, pH 6.0), yeast nitrogen base (1.34%), biotin (4×10^{-4} g L^{-1}) and glycerol (1%)); BMMY (BMGY but using methanol (0.5%) instead of glycerol). The media were supplemented with zeocin (100 mg L^{-1}). Cells were cultivated in baffled flasks at 30 °C and 225 rpm.

The expression vector pPICZ A (Invitrogen) was used for the initial cloning steps and for expression of CYP2D6 and CPR under control of the alcohol oxidase (AOX1) promoter in *P. pastoris*. Plasmids harbouring genes encoding CYP2D6 (SK+2D6(374V)) and human NADPH-oxidoreductase (hOR-PAK9), were kindly provided by Dr. U. Zanger (Institute of Clinical Pharmacology IKP, Stuttgart, Germany).

Acetylcholinesterase from *Nippostrongilus brasiliensis* expressed in *P. pastoris* was a gift from Dr. H. Schulze (ITB, Stuttgart, Germany).

Recombinant DNA technologies: Standard DNA technologies were used unless stated otherwise.[35] The Genelute Plasmid Mini-Prep Kit (Sigma), the QIAPrep Midi Plasmid preparation kit (Qiagen, Hilden Germany), and the NucleoSpin Extract Kit (Machery & Nagel, Düren, Germany) were used for plasmid DNA and DNA gel extractions, respectively. Restriction enzymes and other DNA-modifying enzymes were used as specified by the supplier (MBI Fermentas (St. Leon-Rot, Germany)). DNA-sequencing reactions were carried out on both strands of double-stranded templates by using the BigDye Terminator Cycle Sequencing Kit RR-100 (Applied Biosystems, Weiterstadt, Germany). The sequencing products were analysed on a ABI Prism 377 DNA Sequencer (Perkin–Elmer, Shelton, USA). Standard protocols were used for the preparation and transformation of competent *E. coli* cells.[35] Transformation of *P. pastoris* was carried out according to the Invitrogen electroporation method (Invitrogen). The integration of the *Pme*I linearised vectors into the genome of *P. pastoris* clones was confirmed by using gene specific primers and genomic DNA as template. Genomic DNA was prepared according to the Invitrogen manual (Invitrogen).

Construction of expression vectors: The genes encoding CYP2D6 and CPR were amplified by PCR from plasmids SK+2D6(374V) and hOR-PAK9, respectively, by using two gene-specific primers for each gene (forward primer CYP2D6: 5′-AACCG*GAATTC*ATGGGGCTAGAAGCACTGG-3′, initial codon underlined, *Eco*RI site italicised; reverse primer CYP2D6: 5′-AACCG*CTCGAGCTA*GCGGGGCACAGCACAAAG-3′, stop codon underlined, *Xho*I site italicised; forward primer CPR 5′-AACCG*GAATTC*ATGATCAACATGGGAGACTC-3′, initial codon underlined, *Eco*RI site italicised; reverse primer CPR (5′-AACCG*CTCGAGCTA*GCTCCACACGTCCAG-3′, stop codon underlined, *Xho*I site italicised.

Both PCR products were double digested with *Eco*RI and *Xho*I, gel purified and each ligated into the respective sites of the *P. pastoris* expression vectors pPICZ A (Invitrogen) and pPICZA-ΔPme generating the vectors pPICZ-2D6, pPICZ-CPR, pPICZ-2D6-ΔPme and pPICZ-CPR-ΔPme. pPICZ-ΔPme was previously obtained by site-directed mutagenesis by using the Quik Change Mutagenesis Kit (Stratagene, La Jolla, USA) and the primers A (5′-CCAAAACTGACAGTTTAGACGCTGTCTTGGAACC-3′) and B (5′-GGTTCCAAGACAGCGTCTAAACTGTCAGTTTTGG-3′; altered nucleotides underlined) according to the Kit manual.

To combine both expression cassettes into a single vector, a strategy based on the specificity of the *Bam*HI and *Bgl*II restriction enzymes recognising different sequences, but producing compatible sticky ends was used.[12] The expression cassette of CYP2D6 in pPICZ-2D6 was isolated by double digestion with *Bam*HI and *Bgl*II followed by gel extraction and ligated into pPICZ-CPRΔPme linearised either with *Bam*HI or *Bgl*II, respectively, to give the vector pPICZ-2D6-CPR.

Expression of recombinant proteins in *P. pastoris*: Recombinant clones selected on zeocin plates were picked and grown in BMGY medium (10 mL) to an OD_{600} of 5–10. The cells of this preculture were then collected by centrifugation (5 min, 3000 *g*, room temperature) and used to inoculate BMMY medium (50 mL) in a baffled flask (500 mL) to an OD_{600} of ~1. Induction of the recombinant protein expression in the main culture was performed and maintained by daily addition of methanol (0.5%). After 90 h, cells were harvested by centrifugation (3000 *g*, 10 min, 4 °C).

Preparation of membrane fractions of *P. pastoris*: Cells were washed in homogenisation buffer (potassium phosphate (50 mM, pH 7.9), EDTA (1 mM), glycerol (5%), DTT (2 mM), phenylmethylsulfonyl fluoride (1 mM)) and resuspended to an OD_{600} of 130. The cell suspension was mixed with an equal volume of acid-washed glass beads (0.5–0.75 mm in diameter). Disruption and isolation of microsomes was carried out similarly to the procedure previously described.[26] Cells were disrupted by vortexing (8×30 s at 4 °C with cooling on ice for 30 s between the cycles). The lysate was separated from cell debris and glass beads by centrifugation (12 000 *g*, 10 min, 4 °C). The supernatant was ultracentrifuged (100 000 *g*, 1 h, 4 °C), the microsomal pellet was then resuspended in homogenisation buffer and stored at −80 °C.

Quantification of cytochrome P 450: Protein concentrations were determined by using the BC Assay (Uptima Interchim, Montluçon, France), according to the supplier's recommendation. BSA was used as a standard.

CYP2D6 concentrations in the isolated membranes were determined by reduced carbon monoxide spectra as follows:[36] microsomes (100 μL, 15–30 μg protein per μL) in sodium phospate buffer (0.1 M, pH 7.4) containing glycerol (10%) and Triton X 100 (0.5%) were incubated on ice for 10 min. Some sodium dithionite was added, and insoluble particles were removed by centrifugation (3 min, 14 000 rpm) using a microcentrifuge. The supernatant was transferred into UV cuvettes, and a reference spectrum was recorded from 400 to 500 nm (Ultrospec 3000 UV/Visible spectrophotometer, Pharmacia Biotech). The solution was then aerated with carbon monoxide for 30 s, and the spectrum was measured again.

The cytochrome P450 concentration was calculated with an extinction coefficient of $\varepsilon_{450-490nm}=91$ mM^{-1}cm^{-1} (pmol μL^{-1} cytochrome P450 $=\Delta mOD_{450-490nm}\times$ dilution factor $\times\varepsilon^{-1}\times1$ cm).

SDS-PAGE/Western blotting: The microsomal fraction (10 μL) was separated by SDS-PAGE by using polyacrylamide gels (12.5%) with a stacking gel (4%) under reducing conditions.[37] Specific detection of the heterologous proteins was achieved by Western blotting with CPR- and CYP2D6-specific antibodies purchased from BD Gentest (catalogue numbers 458246 and 299247): After equilibration of gel, Biotrace NT nitrocellulose membrane (Pall GmbH, Dreieich, Germany) and filter sheets in transfer buffer (Tris (25 mM), glycine (142 mM), methanol (20%)), the protein bands were electro-

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blotted onto the nitrocellulose membrane (Pall GmbH) at 15 V for 24–30 min. After the membrane had been washed twice in TBS buffer (Tris/HCl (50 mM, pH 7.5), NaCl (150 mM)), it was blocked 2× 30 min in blocking solution (TBS buffer supplemented with BSA (3%) or skimmed-milk powder (5%), respectively).

After this blocking step, the membrane was washed for 2×10 min in TBST buffer (TBS supplemented with Tween 20 (0.1%)), then 10 min in TBS buffer and probed with the primary antibody (*anti*-CYP2D6 or *anti*-CPR) for 1 h at RT. After being washed as described, the membrane was probed for 1 h at RT with the secondary antibody conjugated with alkaline phosphatase (*anti*-mouse-IgG-AP or *anti*-rabbit-IgG-AP, respectively; Sigma) diluted 1:1000 in TBS buffer. Bound antibodies were detected on the washed membrane (4× with TBST buffer) by incubation in substrate solution ((10 mL), $MgCl_2$ (5 mM), NaCl (100 mM), Tris/HCl (100 mM, pH 9.5) mixed with NBT stock solution (66 µL, NBT (5% (*m*/*v*)) in dimethyl formamide (DMF, 70%)) and BCIP stock solution (33 µL, BCIP (5% (*m*/*v*)) in DMF). The substrate reaction was stopped by incubation in ddH_2O, then the mixture was dried and scanned.

CYP2D6 activity tests

De-ethylation of 3-cyano-7-ethoxycoumarin: The de-ethylation of 3-cyano-7-ethoxycoumarin was measured according to Favreau with modifications,[38] substrate solution (100 µL, 3-cyano-7-ethoxycoumarin (100 µM) in potassium phosphate buffer (100 mM, pH 7.4), NADPH (3 mM), Pluronic F-68 (0.02%)) was preincubated in a microtiterplate well at 37°C for 5 min and mixed with microsomal protein (300 µg) in potassium phosphate buffer (100 mM, pH 7.4). Samples were excited at 405 nm, and emission at 460 nm was detected and recorded every 10 min for a total of 160 min on a FluoStar Fluorimeter (BMG Lab Technologies, Offenburg, Germany).

Coupled acetylcholine esterase–CYP2D6 assay: The assay is based on the inhibition of the acetylcholine esterase by paraoxon, as described by Sams.[39] Initially parathion is activated in a CYP2D6-catalysed reaction to paraoxon. Therefore, a solution of parathion (25 µL, 20 $\mu g\,mL^{-1}$) in potassium phosphate buffer (50 mM, pH 7.5) was mixed with microsomal proteins (300 µg) and made up to 75 µL with phosphate buffer. After 6 min of incubation, aqueous NADPH (25 µL, 5 $mg\,mL^{-1}$) was added, followed by 40 min of incubation. Potassium phosphate buffer (690 µL, 50 mM, pH 7.4), DNTB (100 µL, 7.8 mM in potassium phosphate buffer) and acetylcholine esterase solution (100 µL) were added to the inhibition mixture. After 30 min of incubation, the enzyme reaction was started by the addition of acetylthiocholine iodide (10 µL, 100 mM). Acetylcholine esterase activity was recorded and expressed as a percentage of the control activity measured without paraoxon (residual activity), as reported by Ellman.[40] The absorption at 412 nm was detected by using a UV/Vis ultrospec 3000 photometer (Pharmacia Biotec, Freiburg, Germany).

Dextromethorphan O-demethylation analysis by HPLC and LC/MS

HPLC: Microsomal protein solution (25 µL, 120 µg in potassium phosphate buffer (50 mM), EDTA (1 mM, pH 7.9), glycerine (5%)) was preincubated for 5 min at 37°C with potassium phosphate (100 mM), EDTA (55 µL, 1 mM, pH 7.4) and dextromethorphan (10 µL, 50 µM in methanol (0.0025%)). The reaction was started by the addition of NADPH (10 µL, 10 mM) and incubated for 60 min at 37°C. The reaction was stopped by the addition of HCl (10 µL, 2 M) and cooled on ice for 5–10 min.

The product was extracted by addition of water-saturated ethyl acetate (660 µL) and vortex mixing (2 min). The organic layer was collected after centrifugation (5 min, 1000 *g*), and the ethyl acetate was removed under a nitrogen stream. The dried product was dissolved in acetonitrile/water/acetic acid/triethylamine (100 µL, 35:65:1:0.02, *v*/*v*/*v*/*v*).

Samples were analysed by high-performance liquid chromatography (HPLC) on a C8 column (4.6×250 mm) in a Shimadzu device with acetonitrile/water/acetic acid/triethylamin (35:65:1:0.02, *v*/*v*/*v*/*v*) as the mobile phase (1 $mL\,min^{-1}$). Dextromethorphan and its metabolites were detected by using a fluorescence detector at excitation and emission wavelengths of 235 nm and 310 nm, respectively.

LC-MS/MS in brief: Dextromethorphan-*O*-demethylase activity was measured with CYP2D6 (5.5 pmol) in sodium phosphate buffer (final volume of 0.25 mL, 0.1 M, pH 7.4) and dextromethorphan (0.1–20 µM) as substrate. After the reaction mixture had reached equilibrium (37°C, 3 min), enzyme reactions were initiated by the addition of an NADPH-regenerating system ($MgCl_2$ (5 mM), glucose 6-phosphate (4 mM), $NADP^+$ (0.5 mM) and glucose 6-phosphate dehydrogenase (4 $U\,mL^{-1}$)). The reaction was stopped by the addition of HCl (50 µL, 1 M).

After addition of the internal standard [D_3]dextrorphan (100 pmol), the samples were mixed and centrifuged (16000 *g*, 5 min). The supernatant was directly injected into the HPLC system. The metabolite dextrorphan was separated and detected by HPLC-MS/MS spectrometry by using a HPLC system (HP 1100, Agilent Technologies, Waldbronn, Germany) equipped with a Omnispher-C18 column (150×3 mm, 5 µM particle size, Varian, Darmstadt, Germany) and an ion-trap mass spectrometer (HCT plus, Bruker Daltronics, Bremen, Germany). Elution was performed with a gradient of 15% acetic acid (1%)/water and 85% acetic acid (1%)/acetonitrile to 50%/50% over 9.5 min. All incubations were performed in duplicate and in the linear range with respect to microsomal protein and incubation time. Control experiments were carried out in parallel by using denaturated microsomes.

Data were processed by using the software Quant Analysis (Bruker Daltronics). Enzyme kinetic data were analysed by using the program GraphPadPrism v3.0 (GraphPad Software Inc., San Diego, CA).

Reductase activity: The reductase-catalysed reduction of bovine heart cytochrome c at 550 nm was measured essentially as described.[41] A solution of cytochrome c (100 µL, 6.5 $mg\,mL^{-1}$) in potassium phosphate buffer (50 mM, pH 7.5) was mixed with microsomal protein (120 µg) and made up with potassium phosphate buffer to 950 µL. Reactions were started by adding aqueous NADPH (50 µL, 9 $mg\,mL^{-1}$). Activities were measured by using a UV/Vis ultrospec 3000 photometer (Pharmacia Biotec, Freiburg, Germany) and calculated by using an extinction coefficient of 21 $mM^{-1}\,cm^{-1}$. Kinetic data were analysed by using the swift program (Pharmacia). One unit is defined as 1 $\mu mol\,min^{-1}$.

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