Recombinant production of human microsomal cytochrome P450 2D6 in the methylotrophic yeast *Pichia pastoris*

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Abstract:
Microsomal cytochrome P450 monooxygenases of the groups 1 – 3 are mainly expressed in liver and play a crucial role in phase 1 reactions of the xenobiotics metabolism. The cDNAs encoding human CYP2D6 and human NADPH-P450 oxidoreductase (CPR) were transformed into the methylotrophic yeast *Pichia pastoris* and expressed under control of the methanol inducible AOX1
promotor. 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 using isolated microsomes. Nearly all the recombinant CYP is composed of the active holo-enzyme as confirmed by reduced CO-difference spectra which showed a single peak at 450 nm. Only by co-expression of human CPR and CYP, CYP2D6 activity was obtained. Microsomes containing human CPR and CYP2D6 converted different substrates, such as 3-cyano-7-ethoxycoumarin, parathion, and dextrometorphan. The kinetic parameters of the dextrometorphan conversion closely matched those of CYP2D6 from other recombinant expression systems as well as from 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 characterization of single microsomal cytochromes as well as their allelic variants.

keywords

Activity, cytochromes, oxidoreductase, xenobiotics, *Pichia pastoris*

Introduction:

Mammalian cytochrome P450 monooxygenases (CYP; E.C. 1.14.14.1) are ubiquitous proteins: on the one hand they are involved in the oxidation of several endogenous compounds such as steroids, prostaglandins and fatty acids; on the other hand CYPs of the groups 1, 2 and 3 play a key role in the metabolism of xenobiotics which consists of two phases: in phase 1 called *functionalization* 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 allows its efficient excretion.

Although the CYPs are expressed at different levels in many tissues, 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 strongly on the 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).

Characteristic for those microsomal CYPs are broad substrate specificities with individually different phenotypes due to polymorphisms, duplications and different expression levels. 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 using animal models have been replaced by *in vitro* studies using human enzymes from different sources.[1-6] Microsomal preparations or cellular systems such as liver slices and human hepatocytes are used and provide the advantage of a maintained cellular integrity concerning 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 which 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] Anyway, some of them are commercially available. Among the simpler systems mainly baculovirus infected

insect cells are currently used to purify microsomes containing individual mammalian CYPs and accessory proteins (CPR & 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 making the scale-up difficult. Due to the cheap cultivation procedures and media required and the ease of handling, *E. coli* was used as host for the expression of a couple of CYPs as well.[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 *S. cerevisae* combines the ease of handling of prokaryotic systems with the features of eukaryotic systems such as posttranslational processing. Whereas initial experiments did not provide reasonable cytochrome P450 expression levels, the group of Pompon 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] Also 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 as well as secreted expression and the ease of handling, the methylotrophic yeast *Pichia pastoris* has gained in popularity as expression system during the last few years. A couple of proteins have been expressed in very high yields (up to 10 g l^{-1}).[22, 23] Despite additional advantages of *Pichia* over the common baker's yeast such as a glycosilation pattern which is closer to humans 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 expression system for the production and characterization of human microsomal CYPs. CYP2D6, the model enzyme we have chosen is a polymorphically expressed microsomal cytochrome P450,[27] which almost exclusively catalyses the conversion of more than 50 relevant drugs including cardivacular 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] Others genotypes show significant phenotype alterations due to shifted kinetic properties or varied expression levels.[31] Poor metabolizers for example lacking CYP2D6 activity and thus do not have an alternative pathway for the detoxification of respective substrates. 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 each into the vector pPICZ A under control of the AOX1 promotor as described in the methods section. Upon linearisation both vectors pPICZ-2D6 and pPICZ-CPR (fig. 1a) were transformed into *Pichia pastoris* X-33, and chromosomal integration of the heterologous expression cassettes was confirmed by PCR using genomic DNA as template and gene specific primers (data not shown). Since expression levels in recombinant *Pichia* clones tend to show clonal variation 5 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 comprising ~ 20 µg microsomal protein were analysed by western blotting. Bands of the correct size of ~55 kDa (CYP2D6) and ~77 kDa were detected in the lanes of all investigated samples. The respective band corresponding to CYP2D6 is shown in lane 1 of figure 3A exemplarily 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. Non-transformed *Pichia* strains or such strains transformed with pPICZ-2D6 or pPICZA (both without 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 in turn is generated in a CYPD6 catalysed reaction by oxidation of parathion. In parallel a fluorescence assay based on the deethylation of 3-cyano-7-ethoxycoumarin is used for the direct determination of CYP2D6 activity. All

clones expressing CYP2D6 showed ~ 95% residual acetylcholine esterase activity. Thus, no significant CYP2D6 activity could be measured (fig. 4). Also deethylation of 3-cyano-7-ethoxycoumarin (fig. 5) was not obtained using microsomes of these strains. Even a mixture of recombinant *Pichia* microsomes containing human CYP2D6 and human CPR did not result in any activity (fig. 4 & 5) towards neither 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 using both assays (data not shown).

Due to the fact that 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 using a cloning strategy which was recently used for the coexpression of heavy and light chain 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 using CPR- and CYP2D6 specific primers. All tested clones contained both genes (fig. 2).

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 4 investigated clones (fig. 3): Whereas a single strong band of ~ 55 kDa was detected by probing the blot with anti-CYP2D6 antibody (fig. 3a, lanes 3 - 6) a strong band of ~ 77 kDa and a weaker one of ~ 58 kDa was detected when probing the blot with CPR-specific antibodies (fig. 3b, lanes 3 – 6).

Using the modified Ellman test microsomes of the 4 investigated coexpression clones showed significant CYP2D6 activity: the residual acetylcholine esterase activities obtained were between 55 and 40 % (fig. 4, C1 – C4) in contrast to 95 % obtained

with samples from clones expressing CYP2D6 only (fig. 4, 2D6-1 – 2D6-4). With microsomes from clones expressing the oxidoreductase only and clones containing the vector pPICZA (both serving as negative controls) no acetylcholine esterase inhibition was obtained as expected (fig. 4, CPR-1 & -2; pPICZ A). These results were confirmed by two assays based on the conversion of two typical substrates of CYP2D6, namely 3-cyano-7-ethoxycoumarin (fig. 5) and dextrometorphan (fig. 7): only microsomes of the coexpression clones catalysed the deethylation of 3-cyano-7-ethoxycoumarin as shown in a fluorescent assay (fig. 5, 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 (fig. 7). Only microsomes of clones expressing both, CYP2D6 and CPR showed conversion of dextrometorphan (DXM, retention time ~ 19 minutes) to dextrorphan (DP, retention time ~ 8.7 min). Microsomes of clones expressing CYP2D6 only or negative controls did not show any product peak.

In oder 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 or 0.12 nmol / mg total protein. The specific activity of the demethylation of dextrometorphan resulted in ~ 5 pmol DP / pmol CYP / min as determined by LC/MS. The kinetic parameters concerning the conversion of dextrometophan (Vmax ~ 8.8 pmol / min / pmol enzyme, K_M ~ 1.9 µM, Vmax/K_M ~ 2.5 µl / pmol enzyme / min) are in the same range like 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 the xenobiotic metabolism. Especially the human ones are needed by many pharmaceutical companies to test drug candidates for adverse drug interactions and thus are of high 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 control of the methanol inducible AOX1 promotor were generated by a strategy previously used for the coexpression of heavy and light chains of a Fab fragment.[32]

Beside zeocin there is no other suitable antibiotic as selection marker for *P. pastoris* commonly in use. Only several auxotrophy markers are used, but due to their low efficiency the screening of a high number of colonies is generally required 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 co-expression 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 pre-treatments such as extractions and there is no need for complicated and expensive devices such as HPLC, GC/MS, LC/MS. The substrate parathion is much cheaper than most of the fluorimetric substrates such as derivatives of coumarin. In addition to the detection of CYP activity the assay can be used for the monitoring of phosphorothionate pesticides as we recently have published elsewhere.[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 concerning the conversion of dextrometorphan. The expression level in *P. pastoris* (120 pmol CYP2D6 / mg protein) is in the same range like that obtained in Baculovirus transformed insect cells and *E. coli*, but significantly higher than HepG2 and other mammalian systems. However, these comparisons lack of significance due to the different and imprecise methods (western blotting, reduced CO-difference spectrum) used for the determination of microsomal CYPs in the different reports. Using the same protocol for the demethylation of dextromethorphan, microsomes

from recombinant *P. pastoris* showed five fold higher substrate conversion rates (4.8 pmol / min / pmol protein) than microsomes from Baculovirus transformed insect cells purchased commercially from BD Gentest (1 pmol / min / pmol protein) as determined by LC/MS.

Expression of CYP2D6 without the coexpression of human CPR was possible as well 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*: Especially the endogenous genes encoding a NADPH-cytochrome P450 reductase, a NADH-cytochrome b5 reductase, and a cytochrome b5 have to be mentioned in this context. These results are in contrast to other studies, which reported that endogenous electron transfer systems of other yeasts are fully compatible with human CYPs: Bureik and colleagues reported that the inner mitochondrial electron transfer system of *Schizosaccharomyces 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, probably this is 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 range of grams per litre. Although some CYPs from spiny lobster, spiny dogfish shark and cassava have been expressed in *P. pastoris* so far,[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: e.g. coexpression of enzymes of the phase 2 reactions with respective CYPs are of relevance when simulating complete degradation pathways of certain xenobiotics as it occurs in mammalian cells or tissues. However, simulating the in vivo sytem 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.

In contrast to the mammalian or viral expression systems which are currently used in most cases for the production of recombinant mammalian CYPs (see table 1), the

yeast *Pichia pastoris* provides an easier to handle and much cheaper alternative. Due to this 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 using whole cells avoiding the isolation of microsomes which currently is the limiting step (will be published elsewhere). Currently this is done using recombinant *E. coli*, but as described above only with N-terminally modified CYP-variants.[11, 12] Thus, such biotransformations using *Pichia* strains coexpressing phase 1 and 2 enzymes would raise the possibility of a biotechnological production of metabolites on a bigger scale and might serve as an alternative to chemical synthesis.

The high substrate conversion rates in combination with the ease of handling including transformation, cultivation and upscaling of *Pichia* is superior to other systems such as CHO-cells, hepatocytes, baculovirus-transformed insect cells but also yeast systems like *Schizosaccharomyces pombe and Saccharomyces cerevisiae*.

Experimental Section

Chemicals

Unless stated otherwise all chemicals were purchased from Sigma-Aldrich Chemie (Steinheim, Germany), Fluka (Buchs, Germany), Merck (Darmstadt, Germany), 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, Goettingen, 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 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 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 (1M)); 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) promotor 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 GmbH & Co KG, Düren, Germany) were used for plasmid DNA and DNA gel extractions, respectively. Restriction enzymes and other DNA modifying enzymes were used as specified from the supplier (MBI Fermentas (St. Leon-Rot, Germany)). DNA-sequencing reactions were carried out on both strands of double-stranded templates 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 done according to the Invitrogen electroporation method (Invitrogen). The integration of the *Pme* I linearized vectors into the genome of *P. pastoris* clones was confirmed 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, using two gene-specific primers for each gene (forward primer CYP2D6: 5´-AACCG*GAATTC*ATGGGGCTAGAAGCACTGG-3´, initial codon underlined, *Eco* RI site italicized; reverse primer CYP2D6: 5´-AACCG*CTCGAG*CTAGCGGGGCACAGCACAAAG-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*CTCGAG*CTAGCTCCACACGTCCAG-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 using the Quik Change Mutagenesis Kit (Stratagene, La Jolla, USA) and the primers A (5'-

CCAAAACTGACAGTTTAGACGCTGTCTTGGAACC-3'; altered nucleotide underlined) and B (5'-GGTTCCAAGACAGCGTCTAAACTGTCAGTTTTGG-3'; altered nucleotide underlined) according to the manual of the Kit.

To combine both expression cassettes into a single vector, a strategy based on the specificity of the *Bam* HI and *Bgl* II restriction enzymes recognizing 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 linearized either with *Bam* HI or *Bgl* II, respectively, resulting in the vector pPICZ-2D6-CPR.

Expression of recombinant proteins in *P. pastoris*

Recombinant clones selected on zeocin plates were picked and grown in 10 ml BMGY medium 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} ~ 1. Induction of the recombinant protein expression in the main culture was performed and maintained by daily addition of methanol (0.5 %). After 90 hours 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); PMSF (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 done similar as previously described:[26] Cells were disrupted by vortexing (8x 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 (12000 g, 10 min, 4°C). The supernatant was ultracentrifuged (100000 x g, 1 h, 4°C), the microsomal pellet was then resuspended in homogenisation buffer and stored at -80°C.

Quantification of cytochrome P450

Protein concentrations were determined using the BC Assay (Uptima interchim, Montluçon, France) according to the supplier's recommendation. BSA was used as standard.

CYP2D6 concentrations in the isolated membranes were determined by reduced carbon monoxide spectra as follows:[36] microsomes (100 µl, 15 – 30 µg protein / 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, 14000 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 afterwards the spectrum was measured again.

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

SDS-PAGE / Western Blotting

10 µl of the microsomal fraction were separated by SDS-Page 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 using CPR-specific and CYP2D6 specific antibodies purchased from BD Gentest (catalog numbers 458246 and 299247): After equilibration of gel, Biotrace™ NT nitrocellulose membrane (Pall GmbH), and filter sheets in transfer buffer (Tris (25 mM), glycine (142 mM), methanol (20 %)) the protein bands were electroblotted onto the nitrocellulose membrane (Pall GmbH, Dreieich, Germany) at 15 V for 24 – 30 minutes. After washing the membrane two times in TBS buffer (Tris/HCl (50 mM, pH 7.5), NaCl (150 mM)) it was blocked 2x 30 minutes in blocking solution (TBS buffer supplemented with BSA (3%) or skim milk powder (5%), respectively).

After this blocking step the membrane was washed 2x 10 minutes in TBST buffer (TBS supplemented with Tween 20 (0.1 %)), 10 minutes in TBS buffer and probed with the primary antibody (anti-CYP2D6 or anti-CPR) for one hour at room temperature. After washing as described the membrane was probed for one hour at room temperature 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 (4x 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, dried and scanned.

CYP2D6 activity tests

Deethylation of 3-cyano-7-ethoxycoumarin

The deethylation 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 pre-incubated in a microtiterplate well at 37°C for 5 minutes 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 minutes for a total of 160 minutes using 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 catalyzed reaction to paraoxon. Therefore, a parathion solution (25 µl, 20 µg ml^{-1} in potassium phosphate buffer (50 mM, pH 7.5) were mixed with microsomal proteins (300 µg) and topped up to 75 µl with phosphate buffer. After 6 minutes of incubation, aqueous NADPH solution (25 µl, 5 mg ml^{-1}) were added followed by 40 minutes 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 incubation for 30 minutes the enzyme reaction was started by 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 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 %)) were pre-incubated for 5 minutes at 37°C with potassium phosphate (100 mM) and EDTA (55 µl, 1 mM, pH 7.4) and dextromethorphan (10 µl, 50 µM in methanol (0.0025 %)). The reaction was started by addition of NADPH (10 µl, 10 mM) and incubated for 60 minutes at 37°C. The reaction was stopped by addition of HCl (10 µl, 2 M) and cooled on ice for 5-10 minutes.

The reaction product was extracted by addition of water-saturated ethyl acetate (660 µl) and vortex-mixing (2 minutes). The organic layer was collected after centrifugation (5 minutes, 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)).

Samples were analysed by high-performance liquid chromatography (HPLC) using a C8 column (4.6 x 250 mm) in a Shimadzu device and acetonitrile:water:acetic acid:triethylamin (35:65:1:0.02; (v/v)) as mobile phase (1 ml min^{-1}). Dextromethorphan and its metabolites were detected 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) using dextromethorphan (0.1 -20 µM) as substrate. After equilibrating the reaction mixture (37°C, 3 min), enzyme reactions were initiated by the addition of NADPH regenerating system ($MgCl_2$ (5 mM), glucose 6-phosphate (4 mM), $NADP^+$ (0.5 mM) and glucose 6-phosphate dehydrogenase (4 U/ml)). 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 (16,000 g, 5 min). The supernatant was directly injected into the HPLC-system. The metabolite dextrorphan was separated and detected by HPLC-MS/MS spectrometry using a HPLC system (HP 1100, Agilent Technologies, Waldbronn, Germany) equipped with a Omnispher-C18 column (150x3 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 % (1 % acetic acid/water) and 85 % (1 % acetic acid/acetonitrile) to 50 %/50 %

from 0 to 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 using denaturated microsomes.
Data were processed using the software Quant Analysis (Bruker Daltronics). Enzyme kinetic data were analyzed using the program GraphPadPrism v3.0 (GraphPad Software Inc., San Diego, CA). Details will be published elsewhere.

Reductase Activity

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

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Legends

Fig 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 trancscription termination region: native transcription termination and polyadenylation signal from AOX1 gene, permits mRNA processing, CPR: sequence encoding human oxidoreductase, CYP2D6: sequence encoding CYP2D6, pUC ori: origin of replication functional in *E. coli*, CYC1 TT: transcription termination region from *S. cerevisiae* allows processing of *Sh* ble mRNA. The expression cassette of CYP2D6 is shown in grey, the one of CPR in white.

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

Fig. 3: Western blot analysis of microsomes taken 90 hours after induction from cultures of *P. pastoris* X-33 transformed with pPICZ-CYP2D6 and pPICZ-CPR-CYP2D6, respectively. Proteins were detected with anti-CYP2D6- (A) and anti-CPR-antibodies (B). 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 9-10: dilution (1:10) of a commercial preparation of CYP2D6 and oxidoreductase recombinantly expressed in *E. coli* as positive control (New England Biolabs).

Fig. 4: The CO-difference spectrum of recombinant CYP2D6 clearly shows only one peak at 450 nm corresponding to the correctly folded holo-enzyme.

Fig 5: Ellman test for the detection of CYP2D6 activity by inhibition of recombinant acetylcholine esterase. Microsomes were isolated 90 hours 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 (negative control, grey), pPICZ A:

microsomes of *P. pastoris* X-33 harbouring pPICZ A (negative control, grey), 2D6 +CPR: mixtures of microsomes from clones containing pPICZ-CPR or pPICZ-CYP2D6.

Fig 6: Deethylation of 3-cyano-7-ethoxycoumarin was measured using a fluorescence assay (excitation at 405 nm, emission at 460 nm). Microsomes of recombinant *Pichia* strains were isolated after 90 hours of induction. F: fluorescence; C1-C4 microsomes of coexpression clones; 2D6-1 & 2D6-2 microsomes of clones expressing CYP2D6; CPR- microsomes of clones expressing CPR, pPICZA microsomes of a clone transformed with pPICZ A (negative control), 2D6 + CPR mixtures of microsomes of clones expressing CYP2D6 and CPR.

Fig. 7: 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 control, grey)

Fig. 8: The demethylation of 5 µM dextrometorphan by CYP2D6 containing microsomes isolated from a *P. pastoris* X-33 harbouring pPICZ-CPR-CYP2D6 90 hours after induction was measured by HPLC. The output of the fluorescence signal (FS) is in voltage (V) due to a potential transformer integrated in the system.

Table of contents

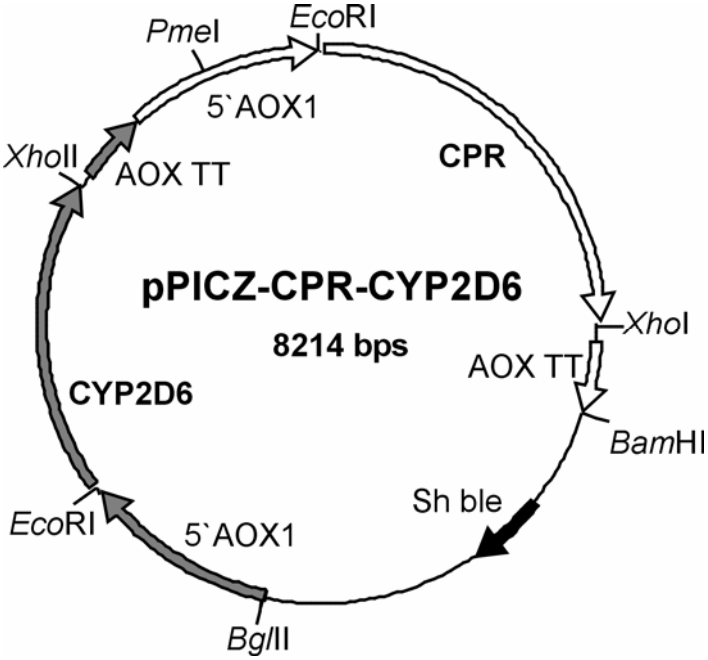
Metabolites of drugs and other xenobiotics generated by cytochrome P450 catalysed phase 1 reactions in liver often decide on the success or failure of novel drug candidates. The methylotrophic yeast *Pichia pastoris* is able to produce active human cytochrome P450 2D6 by coexpression with the NADPH P450 oxidoreductase (see vector map). It thus allows the generation of kinetic data to be used in systems biology of hepatocytes as well as the production of metabolites by biotransformation.

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

Host system	Purific.	Concentration pmol/mg protein	KM µM	Vmax pmol/pmol/min	Vmax/KM µl/pmol/min	References
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/transfection	5.4	0.68	0.13	[31]
HepG2	micros. lysate	35 - 45 15 - 25	n.d.	n.d.	n.d.	[45]
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/min	n.d.	[49]

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

Figure 1:



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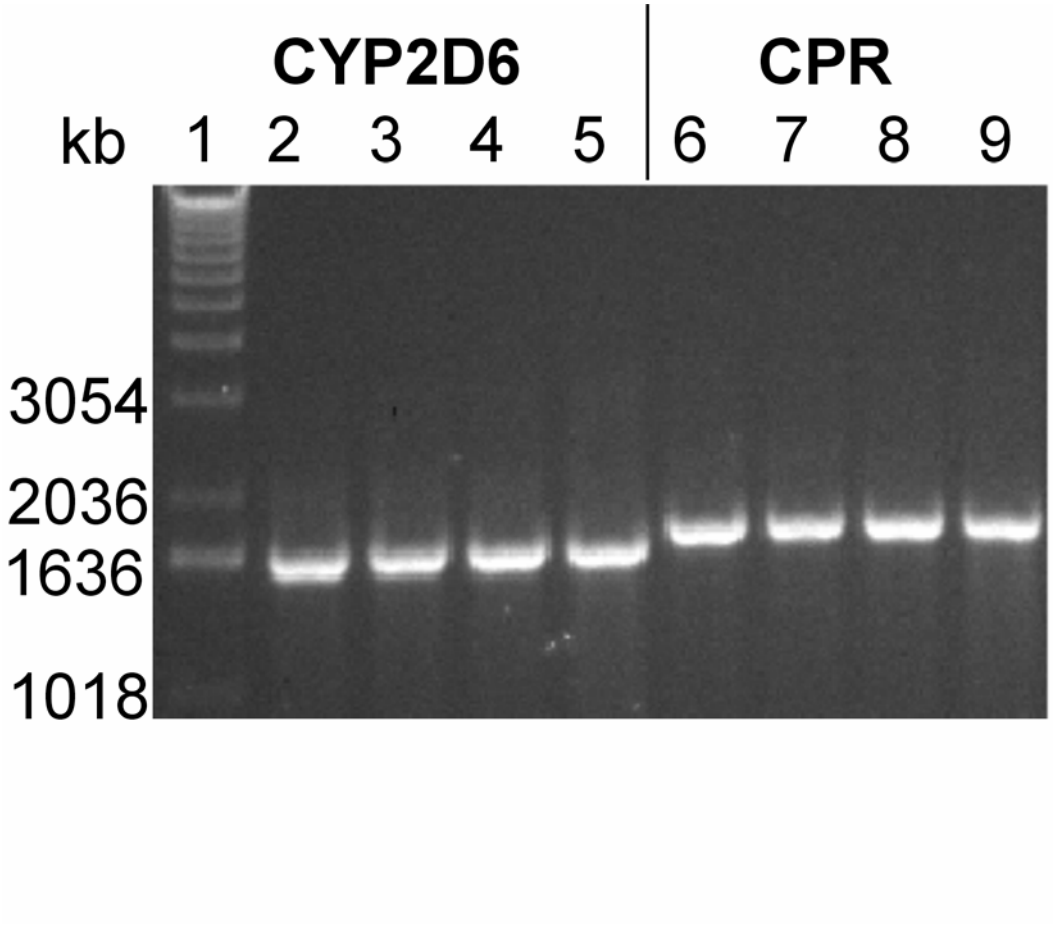

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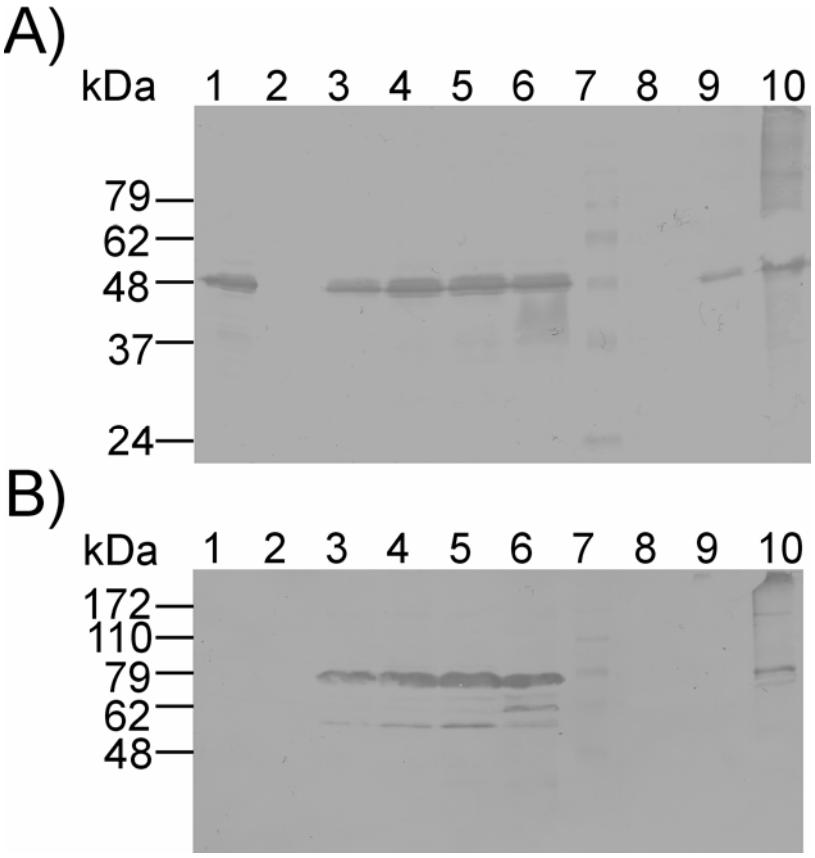

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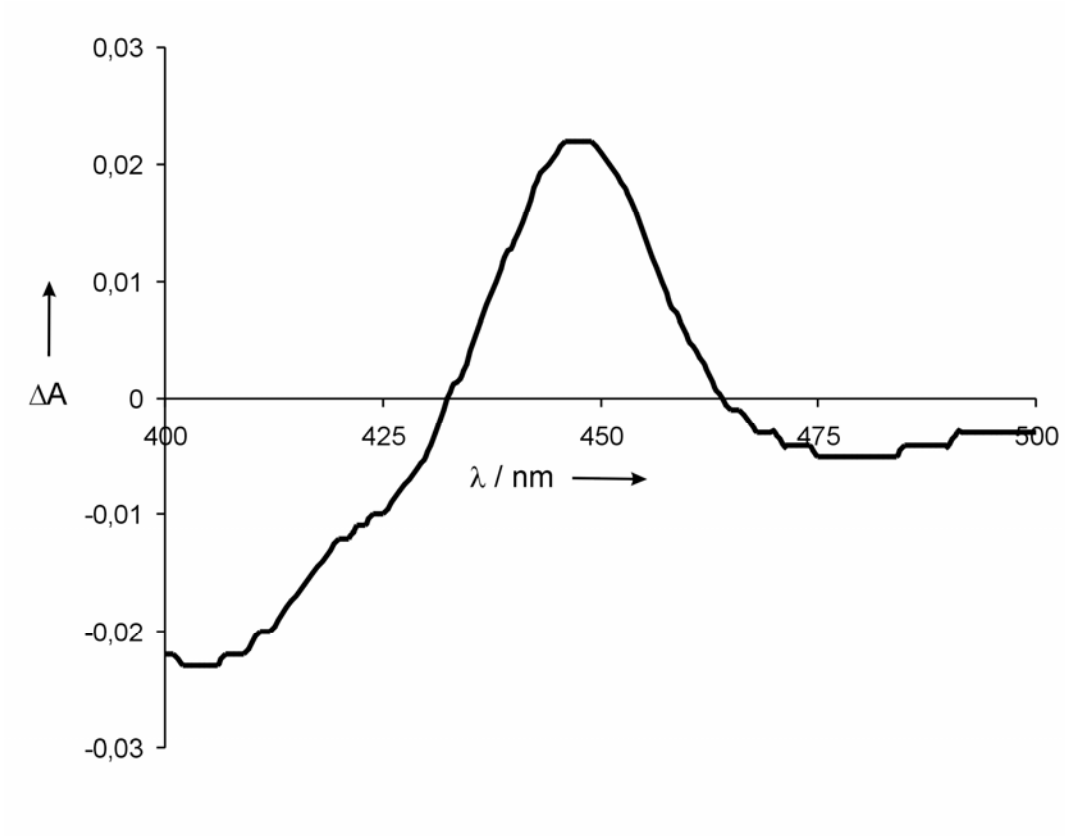

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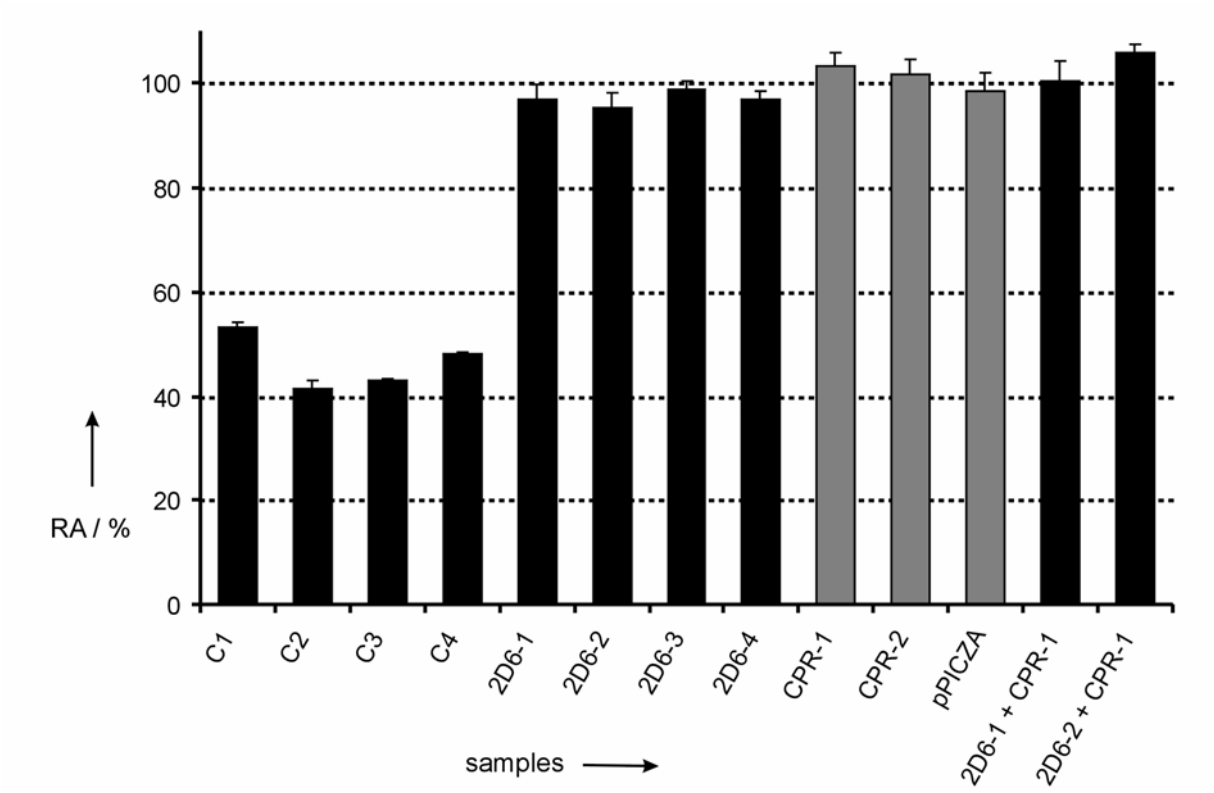

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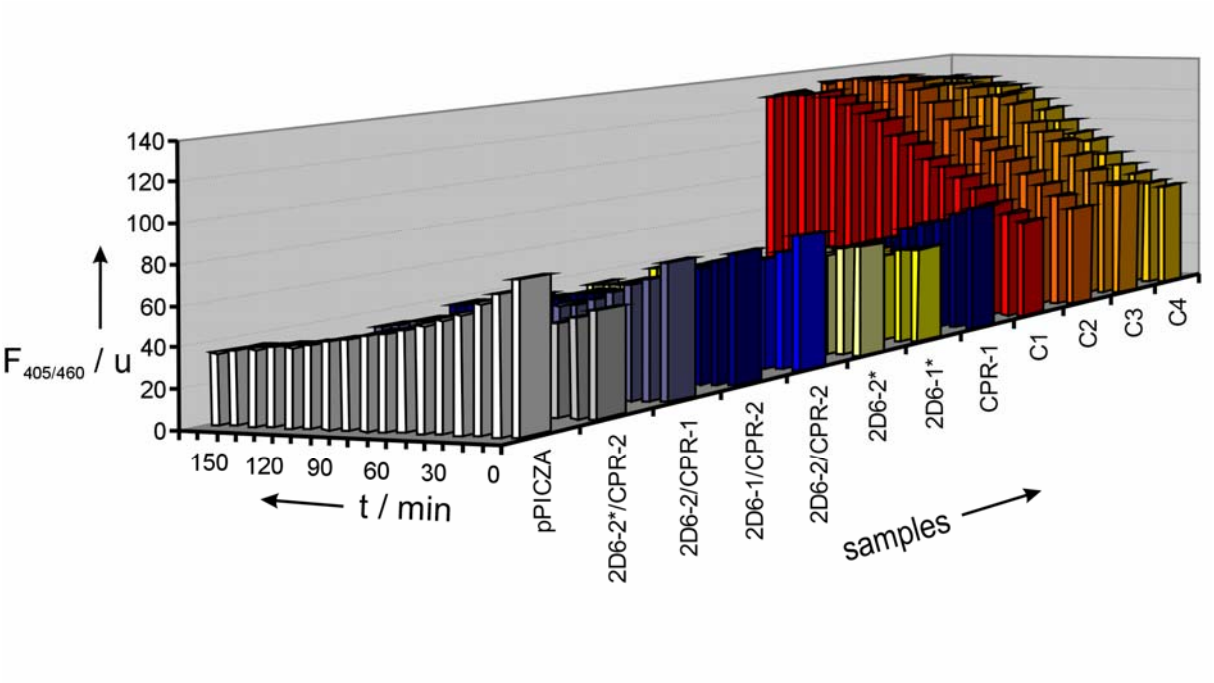

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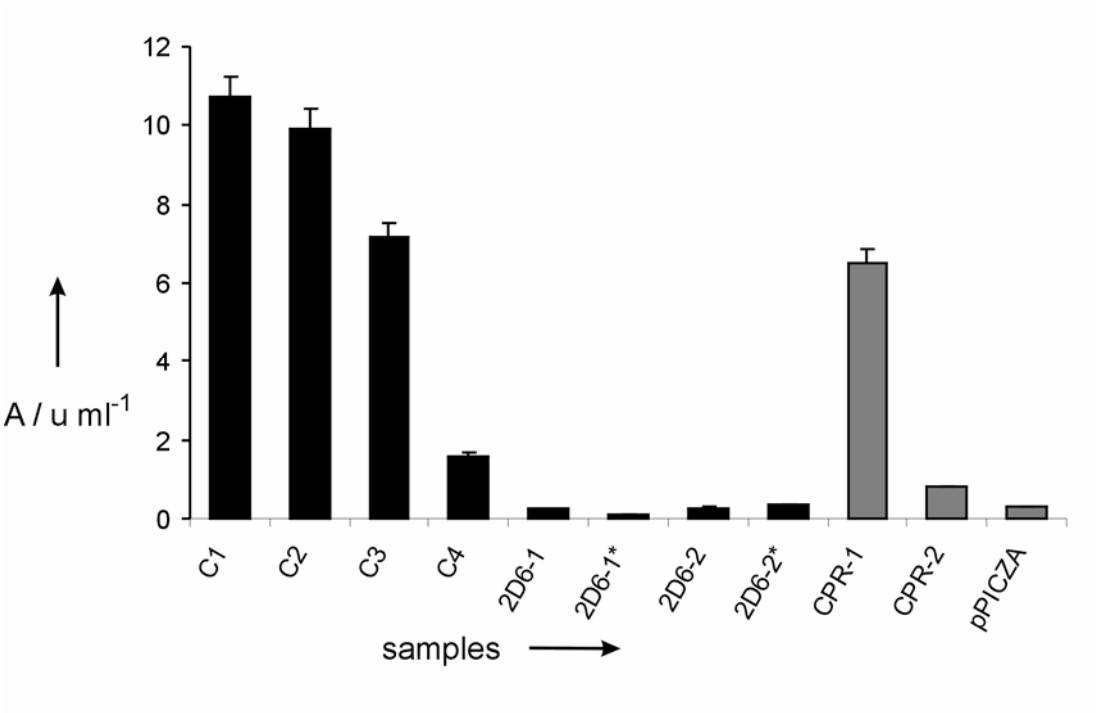

Figure 8: