Biotechnological synthesis of drug metabolites using P450-expressing fission yeast – new perspectives in metabolism studies for 4’-methyl-a-pyrrolidinobutyrophenone

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AIMS: Reference standards of drug metabolites are needed for their structural confirmation and pharmacologic/toxicologic characterization, including studies on their pharmacodynamic/kinetic properties, on enzyme kinetics of their formation, and on phase II metabolism. However, such metabolite standards are often not commercially available. Their classical chemical synthesis can be cumbersome and stereochemically demanding. Therefore, one aim of this study was evaluating the feasibility of biotechnological synthesis of drug metabolites. Human CYP2D6 heterologously expressed in fission yeast Schizosaccharomyces pombe was used as model enzyme and the designer drug 4’-methyl-a-pyrrolidinobutyrophenone (MPBP) as model drug. Another aim was to confirm the structure of the metabolite 4’-hydroxymethyl-a-pyrrolidinobutyrophenone (HO-MPBP) and to use it as a reference standard for further studies.

METHODS: Transformation of fission yeast with integration plasmid pCAD1-CYP2D6 yielded strain CAD49. Correct expression of human CYP2D6 was demonstrated by O-demethylation of dextromethorphan (DXM). For synthesis of HO-MPBP, 250 µmol of MPBP·HNO3 were incubated with 1 L CAD49 culture (10^8 cells/mL, pH 9, 48 h, 30°C). HO-MPBP was isolated from the incubation mixture by liquid-liquid extraction with ethyl acetate and precipitated from the organic phase as hydrochloride. Identity and purity of the product were tested by HPLC-UV, GC-MS, and 1H-NMR. For further characterization of CAD49, the influence of incubation pH (5-10), cell density (10^7-10^8 cells/mL), and incubation time (2-8 h) on metabolite formation were studied using the substrates DXM and MPBP. Reactions were stopped by centrifugation and separation of the supernatants, which were analyzed without further processing by HPLC with UV and fluorescence detection for HO-MPBP and O-demethyl DXM (DXOH), respectively. The synthesized HO-MPBP standard was used to re-evaluate in vitro K_m and V_max values of MPBP 4’-hydroxylation using cDNA expressed CYP2D6, CYP2C19, and CYP1A2 from baculovirus infected insect cells and pooled human liver microsomes (pHLM). Incubations and analyses were performed as described before (Peters et al., TIAFT 2005). In vivo K_m and V_max values of CYP2D6 in CAD49 were determined by incubation of MPBP (10^8 cells/mL, pH 8, 30°C, 30 min) and direct analysis of supernatants by HPLC-UV.
RESULTS: The preparative incubation experiment yielded 40 mg (141 µmol) of HO-MPBP-HCl with a purity of >98%. Its postulated structure was confirmed by 1H-NMR. In the characterization experiments, the metabolite formation rate peaked at pH 8. A linear relationship was observed between cell density and metabolite formation ($R^2 > 0.996$). The rate of metabolite formation was slower in the earlier stages of incubation but then increased. For HO-MPBP, it became constant in the time interval 4.5-8 h ($R^2 > 0.999$). The in vitro $K_m$ values of MPBP hydroxylation in µM were 2.4 (CYP2D6), 9.2 ($K_{mi}$, CYP 2C19), 283 ($K_{mi}$, CYP2C19), 1674 (CYP1A2), and 174 (pHLM). The $V_{max}$ values in pmol/min/pmol CYP were 0.07 (CYP2D6), 0.14 ($V_{max1}$, CYP2C19), 0.27 ($V_{max2}$, CYP2C19), 0.89 (CYP1A2), and 10.5 pmol/min/mg protein (pHLM). Resulting percentages of the three individual CYPs in the net clearance of MPBP were 55% (CYP2D6), 29% (CYP2C19), and 16% (CYP1A2). These $K_m$ and percentage of net clearance data confirm those determined earlier without reference standard. The in vivo $K_m$ and $V_{max}$ values for CYP2D6 catalyzed MPBP hydroxylation were 17.3 µM and 1.7 pmol/min/10^6 CAD49 cells.

CONCLUSIONS: The fission yeast strain CAD49 expressing human CYP2D6 proved feasible for semi-preparative synthesis HO-MPBP, which was successfully used as reference standard for in vitro and in vivo studies on enzyme kinetics allowing determination of $V_{max}$ values in pmol/min/pmol CYP. The higher in vivo $K_m$ of CYPD6 as compared to the in vitro value indicates transport into the cells may be critical for overall in vivo kinetics. Studies with other human CYPs heterologously expressed in fission yeast are in progress.

KEYWORDS: MPBP, Drug, synthesis, Schizosaccharomyces pombe, Cytochrome P450 enzymes

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