Bupropion hydroxylation as a selective marker of rat CYP2B1 catalytic activity

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Abbreviations

ANF = α-naphthoflavone, BNF = β-naphthoflavone, BROD= Benzyloxyresorufin-O-Dealkylase, BUP=
Bupropion, CYP = cytochrome P450, HBUP= Hydroxybupropion, PB = phenobarbital, RHM = Rat Hepatocyte
Microsome, RLM = Rat Liver Microsome
Abstract

Benzyloxyresorufin-\(O\)-dealkylation (BROD) is usually used as a marker of cytochrome P450 (CYP) 2B1 in rat. However, some reports show that CYP1A2 is also highly implicated. The purpose of the present study was to establish bupropion (BUP) hydroxylation, but not BROD, as a selective \textit{in vitro} marker of CYP2B1 catalytic activity. \(IC_{50}\) for BROD and BUP hydroxylation were equivalent (40.8 ± 4.6 and 41.8 ± 3.4 µM, respectively) when using liver microsomes from \(\beta\)-naphthoflavone (BNF)-pretreated rats in the presence of metyrapone (CYP2B1 inhibitor). When using the same microsomes, in the presence of CYP1A1/2-selective inhibitor \(\alpha\)-naphthoflavone (ANF), we found an \(IC_{50}\) of 2.5x10\(^{-3}\) ± 0.8x10\(^{-3}\) µM for BROD while >100 µM for BUP hydroxylation. These results suggest that CYP2B1 is similarly involved in both activities, whereas CYP1A2 is involved in BROD activity, but not in BUP hydroxylation. BUP hydroxylation was assessed in microsomes from baculovirus-infected insect cells co-expressing NADPH-P450 oxidoreductase and 14 rat CYPs and kinetic parameters (\(K_m\) and \(V_{max}\)) were determined. BUP hydroxylation was predominantly catalyzed by CYP2B1 (75% of total hydroxybupropion formation) and low activity was detected with CYP2E1 and CYP2C11 (10.9 % and 8.7% of total hydroxybupropion, respectively) and activity was almost undetectable with the other CYP isoforms at saturating substrate concentrations (2500 µM), thereby validating the use of BUP as a diagnostic \textit{in vitro} marker of CYP2B1 catalytic activity in rat.
Introduction

In the development of new chemical entities for use as medicines in humans, pre-clinical screening includes investigation of the metabolism rates and routes in the safety evaluation species in order to ensure that the metabolites produced in these species are consistent with those predicted to be produced in humans. Sprague Dawley or Wistar rats are often used as the rodent species in these evaluations. CYPs are the family of heme-containing drug-metabolizing enzymes that are involved in the biotransformation of xenobiotics, environmental contaminants, dietary components, and procarcinogens (Wrighton and Stevens, 1992; Gonzalez and Gelboin, 1994). Rat CYP2B1/2, mouse CYP2B9/10, and human CYP2B6 share approximately 80% nucleotide sequence identity (Lewis et al., 1999). Compared with several other CYP subfamilies, CYP2B enzymes (<1% of total CYP) exhibit a relatively low degree of catalytic preservation across mammalian species (Kedzie et al., 1991; Schenkman and Griem, 1993). Three CYP2B isoenzymes, CYP2B1, 2B2 and 2B3, have been identified in rats (Desrochers et al., 1996), these enzymes being the main hepatic CYP isoform inducible by phenobarbital (PB) and other barbiturates. CYP2B1 and 2B2 are structurally related isoenzymes (sharing a 97% amino acid sequence similarity; Suwa et al., 1985) with very similar substrate specificities (Waxman, 1988). Recently, PB has been reported to induce to a significant extent mRNA expression not only of CYP2B1 (500 fold) but also of CYP3A1 (110 fold), while a much lower induction was observed with other isoforms such as CYP2C6 (4 fold), 2E1 (3 fold) and no induction of CYP2C11 mRNA expression was observed (Caron et al., 2005). CYP2B1 is generally much more catalytically active than CYP2B2. Both are expressed constitutively in the liver and extrahepatic tissues such as small intestine and lungs (Lindell et al., 2003). CYP2B enzymes metabolize a diverse group of compounds including pesticides (Dehal and Kupfer, 1994), chemotherapeutics such as cyclophosphamide (Chang et al., 1993), tobacco specific nitrosamines (Stiborova et al., 1996), drug of abuse such as cocaine (Poet et al., 1994), nicotine (Nakayama et al., 1993) and antidepressant (Lewis et al., 1999) including bupropion (BUP).

BUP is an aminoketone used as antidepressant and non-nicotine aid to smoking cessation (Stewart et al., 2001). In humans, BUP is metabolized to hydroxybupropion (HBUP), the pharmacologically active main metabolite (Fig. 1). Previous in vitro reaction phenotyping studies using cDNA-expressed recombinant CYPs or human liver microsomes demonstrated that BUP is metabolized primarily by CYP2B6 to HBUP with an apparent $K_m$ of 85–156 μM in recombinant expressed CYP2B6 and 89–130 μM in human liver microsomes; CYP2E1 and CYP3A4 have also been identified as participating in the metabolism of BUP, but at significantly lower rates than CYP2B6 and only at extremely high BUP concentrations (Faucette et al., 2000, 2001; Hesse et
In rats, it has been shown that following a single intraperitoneal (i.p.) administration of 40 mg/kg or oral administration of 200 mg/kg, BUP is quickly metabolized to HBUP with T_max of 0.68 hr (Suckow et al., 1986; Welch et al., 1987) but the enzyme(s) involved in this metabolic pathway has (have) to our knowledge not been identified. Previously investigated substrate probes of CYP2B1 activity include benzyloxyresorufin-O-dealkylation (BROD) and pentoxyresorufin-O-dealkylation (PROD) in vitro (Burke et al., 1985, 1994; Lubet et al., 1985). However, it has been more recently shown that the dealkylation of benzyloxyresorufin and pentoxyresorufin are, in addition to CYP2B1, catalyzed by multiple enzymes, most notably CYP1A1/2, and to a lesser extent by CYP2C6/11/13, CYP2E1 and CYP3A1/2 (Kobayashi et al., 2002; Chovan et al., 2007). For BROD activity, CYP2B1 showed the highest activity (0.47 pmol/min/pmol CYP), whereas CYP1A2 was also active (0.27 pmol/min/pmol CYP) (Kobayashi et al., 2002). Therefore, alternative catalytic probes of CYP2B1 activity that are more selective would be very useful for in vitro assay. In a previous study (Richert et al., 2009), we found a poor correlation between BROD activity and CYP2B1 mRNA expression in rat hepatocytes after 24 h and 72 h of culture. By contrast, BUP hydroxylation correlated well with CYP2B1 mRNA expression at both time points. The purpose of the present study was to establish BUP hydroxylation, but not BROD, as a selective in vitro marker of CYP2B1 catalytic activity by using liver microsomes from β-naphthoflavone (BNF)- and PB-pretreated rats, in the presence and absence of known CYP inhibitors and a panel of rat cDNA-expressed CYP enzymes.
Methods

Chemicals and reagents

BUP, bovine serum albumin (BSA), benzyloxyresorufin, resorufin, α-naphthoflavone (ANF), BNF, metyrapone, proadifen, reduced nicotinamide adenine dinucleotide phosphate (NADPH), PB, dexamethasone (DEX), 3-methylcholantrene (3-MC), Fenofibrate (FEN) were purchased from Sigma–Aldrich (Saint Quentin Fallavier, France). HBUP and Microsomes prepared from baculovirus-infected insect cells (SUPERSOMES) were purchased from Gentest Corp. (Woburn, MA, USA). NADPH-P450 oxidoreductase was co-expressed in all microsome preparations, and cytochrome b5 was expressed in microsomes containing cDNA-expressed CYP2A1, CYP2A2, CYP2B1, CYP2C6, CYP2C11, CYP2C12, CYP2C13, CYP2E1, CYP3A1 and CYP3A2. The CYP content of each preparation (pmoles of CYP/mg of protein) was spectrophotometrically determined by the supplier as follows: 286 (CYP1A1), 286 (CYP1A2), 196 (CYP2A1), 112 (CYP2A2), 77 (CYP2B1), 250 (CYP2C6), 385 (CYP2C11), 83 (CYP2C12), 143 (CYP2C13), 143 (CYP2D1), 50 (CYP2D2), 208 (CYP2E1), 270 (CYP3A1) and 71 (CYP3A2). Microsomes containing baculovirus vector only served as controls for experiments with cDNA-expressed enzymes. All other laboratory chemicals were used as the highest purity and from commercial suppliers.

Preparation of rat hepatocyte microsomes (RHM)

This study has been carried out in accordance with the guide for the care and use of laboratory animals. Male Wistar rat hepatocytes were isolated as previously described by Richert et al. (2002). Rat hepatocytes (3.5 million cells) were seeded in 60 mm dishes in Human Hepatocyte Maintenance Medium (HHMM) medium (Primacyt, Schwerin, Germany) supplemented with 5% foetal calf serum, gentamycin (50 mg/l), insulin (4 mg/l) and DEX (0.1 μM). Cells were allowed to attach by incubating under a CO2/air (5%/95%) humidified atmosphere maintained at 37°C. After 4 h, the culture medium was replaced with fresh serum-free medium containing test compound. Test compounds were dissolved in serum free HHMM medium supplemented with gentamycin (50 mg/l), Insulin-Transferrin-Selenium-A Supplement, (1x, Cergy Pontoise, France) and DEX (0.1 μM) to give final concentrations of: 5 μM 3-MC, 10 μM BNF, 10 μM DEX, 100 μM FEN or 1000 μM PB. Control cultures were treated with the solvent, DMSO (0.1% v/v final concentration). The medium containing test compound was replaced every 24 h. After 24 h and 72 h of treatment, hepatocytes were harvested in homogenisation buffer and were submitted to several differential centrifugations, as previously described (Richert et al., 2002). The final microsomal pellets were suspended in 0.25 M sucrose. All samples were stored
at −80°C. The protein content was determined using the bicinchoninic acid protein determination kit (Sigma, Saint Quentin Fallavier, France) and BSA was used as a standard. Hepatocyte microsomal enzyme activity determinations were carried out by incubating hepatocyte microsomes with respective probe substrates: BROD (Burke et al., 1985) and BUP hydroxylation (Faucette et al., 2000).

**Preparation of rat liver microsomes (RLM)**

This study has been carried out in accordance with the guide for the care and use of laboratory animals. Male Wistar rats (8 weeks old) were purchased from Janvier (Saint Berthevin, France) and housed in metal cages with a 12 h light/dark cycle and were fed *ad lib* for 48 h. Rats were pretreated with the conventional CYP inducers, the dose and dosing periods employed were as follows: *i.p.* injections of BNF at 40 mg/kg in 0.8 ml corn oil for 3 days, and PB at 80 mg/kg in 0.75 ml corn oil for 3 days. The control rats received 0.8 ml corn oil vehicle only by the daily *i.p.* injections for the same duration. After the last treatment, rats were starved for 24 h prior to sacrifice to reduce the hepatic glycogen content. The rats were sacrificed, and the liver immediately removed and homogenized in 50 mM Tris–HCl, 150 mM KCl, 2 mM EDTA, pH 7.4. The homogenates were submitted to several differential centrifugations, as previously described (Richert et al., 2002). Microsomal samples were frozen at −80°C until analysis. The protein content was determined using the bicinchoninic acid protein determination kit (Sigma, France) and BSA was used as a standard.

**Microsomal assays**

Microsomal BROD was determined according to Burke et al. (1985). Briefly, RLM (0.04 mg protein) were incubated for 2 min at 37°C with benzoyloxyresorufin (20.5 μM) in Tris buffer as substrate in a total volume of 0.1 ml. The reaction was initiated by adding NADPH (1 mM) and was stopped with ZnSO₄ (87 mM) and Ba(OH)₂ (79 mM). Following centrifugation (800 g, 5 min) to remove precipitated protein, the fluorescent metabolite resorufin was measured by spectrofluorometry (excitation 530 nm and emission 580 nm). Calibration standards (1.25–50 pmol/ml) were prepared by adding known amounts of resorufin to microsomes and incubation buffer. Results were expressed as pmol resorufin formed/min/mg microsomal proteins. Rates of HBUP formation were determined using insect cell-derived microsomes and RLMs according to Faucette et al. (2000). Preliminary experiments in RLMs and insect cell-derived microsomes were conducted to identify microsomal protein amounts and incubation times resulting in linear rates of HBUP formation. Incubation mixtures consisted of 0.05 mg of RLMs or 50 pmol of cDNA-expressed CYP enzyme, 25 to 2500 μM BUP,
62.5 mM potassium phosphate buffer (pH 7.4), 1 mM EDTA, 3 mM magnesium chloride, 1 mM NADPH in a total volume of 0.25 ml. All BUP stock solutions were prepared in methanol. Reactions in RLMs were initiated at 37°C by the addition of NADPH and stopped after 30 min with 125 µl of ice-cold acetonitrile. Incubation mixtures were centrifuged at 3000 rpm for 5 min. The supernatant (100 µl) was injected onto the HPLC column. Similar procedures were followed for incubations with microsomes containing cDNA-expressed CYP enzymes, except that reactions were initiated by adding ice-cold microsomes rather than NADPH and then analyzed by LC/MS/MS.

**HPLC Analysis.**

The HPLC system for detection of HBUP consisted of an Agilent 1100 liquid chromatography (Agilent Technologies, Waldbronn, Germany) connected to a Agilent Technologies model 1100 UV detector set at 210 nm. Peak of interest was separated on a 5 µm BDS Hypersil 15x0.46 cm C18 column (Thermo Scientific, France). Mobile phases A (0.1% triethylamine) and B (100% acetonitrile) were pumped at a flow rate of 1 ml/min using a gradient ranging from 10 to 20% B at 0 to 7.5 min, 20 to 50% B at 7.5 to 8 min, 50% B at 8 to 12.5 min and 10% B at 12.5 to 13 min. The column temperature was maintained at 35°C. HBUP peaks were integrated using an Agilent ChemStation system. Retention time for HBUP was approximately 8.7 min. Calibration standards (120 – 2400 pmol/ml) were prepared by adding known amounts of HBUP to microsomes and reagent stock. HBUP concentrations were calculated from the peak area using least-squares linear regression, with weighting by the reciprocal of the squared standard concentrations. Interday coefficients of variation for calibration standards ranged from 12.5% for the lowest standard to 5% for the highest standard. The lower limit of detection was 120 pmol/ml. This concentration is lower than HBUP concentrations observed in rat plasma up to 4 h after 40 mg/kg *i.p.* dose of BUP (Suckow *et al.*, 1986).

**LC/MS/MS Analysis.**

The mass spectrophotometer used was Varian (Les Ulis, France) 320 MS triple quadrupole with pump (Varian 212-LC) and autosampler (ProStar 430). MS Workstation was used for system control and chromatographic data acquisition. The injection volume of samples and standards was 20 µl. The analyses were separated on a Phenomenex Gemini C18 (50 x 2.0 mm, 5 µm) reverse phase column with Gemini security guard cartridge (4x2 mm), at room temperature. The flow rate was 300 µl/min. The mobile phases consisted of A: water, 0.1% formic acid and B: acetonitrile, 0.1% formic acid. The linear gradient was as follows: 0% B from 0
to 1 min, 0% to 97% B from 1 to 4 min, 97% B from 4 to 5 min, and 97% to 0% B from 5 to 5.06 min. The run time was 5 min and the equilibration time between injections was 5 min. The analyse peaks were detected by Mass Spectrometry. Auto-tuning was carried out for maximizing ion abundance followed by the identification of characteristic fragment ions using the generic parameters: CID gas pressure: 2.2 mTorr; ESI needle voltage: 6000 V; drying gas temperature: 350°C; nebulizer gas pressure: 40°psi; drying gas pressure: 50 psi. MRM transitions were monitored in the positive mode as follows: m/z 238.1 → 138.9, ESI, capillary voltage 92 V, collision energy 25 eV, m/z 238.1 → 167.0, ESI, capillary voltage 92 V, collision energy 20 eV. The standard curve range was 10 nM (corresponding to LLOQ) to 2400 nM (corresponding to ULOQ) of HBUP in phosphate buffer. Interday coefficients of variation for calibration standards ranged from 4.15% for the lowest standard to 3.3% for the highest standard. The lower limit of detection was 5 pmol/ml. Activities were expressed as pmol HBUP formed/min/pmol of CYP.

Statistical Analyses

BROD activities in RHM were compared to BUP hydroxylase activities in the same microsomal samples using the linear regression program of GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA). p values for the r-squared (r^2) were determined from an F test. The acceptance limit for statistical significance was set at α = 0.05.

Inhibition of BROD and BUP Hydroxylase Activities by Selective Inhibitors.

The effects of ANF (CYP1A2 inhibitor), metyrapone (CYP2B1 inhibitor), proadifen (CYP1A2/2B1 inhibitor) on BROD and BUP hydroxylase activities were evaluated in BNF-pretreated and PB-pretreated RLMs. The inhibition experiments were conducted with 0.001 to 100 µM of inhibitors at 500 µM BUP for BUP hydroxylation or 20.5 µM benzyloxyresorufin for BROD. This pilot experiment was performed to confirm the ability of metyrapone and proadifen to inhibit the BROD and BUP hydroxylation by using both PB-pretreated RLM and cDNA-expressed CYP2B1. The indicated amounts of inhibitor were pre-incubated on ice with 0.05 mg RLMs for 5 min before reactions were initiated by addition of reagent stock consisting of 500 µM BUP in 62.5 mM potassium phosphate buffer (pH 7.4) or 20.5 µM benzyloxyresorufin in Tris buffer, and 1mM NADPH. Control incubations containing 62.5 mM potassium phosphate buffer or Tris buffer and 1% methanol without inhibitors were performed in parallel. Rates of HBUP or resorufin formation in the presence of inhibitors were expressed as the percentage of control activity. The IC_{50} values for inhibitors were determined by nonlinear
regression analysis of the plot of the logarithm of inhibitor concentration versus percentage of remaining activity using GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA). The enzyme activities in the presence of inhibitors were compared with the control incubation.

Enzyme Kinetic Analyses.

The kinetics of BUP hydroxylation for PB-induced RLMs, and for microsomes containing cDNA-expressed CYP2B1, CYP2C6, CYP 2C11, CYP 2E1 and CYP 3A1 were examined over the concentration range of 25 to 2500 µM. Kinetic parameter estimates were selected by visual inspection of Michaelis-Menten and Lineweaver-Burk of experimental data. Weighted kinetic data were fit to one-component Michaelis-Menten models via iterative nonlinear regression analysis using initial parameter estimates or substrate inhibition model (GraphPad Software, Inc., San Diego, CA, USA). The apparent $K_m$ and $V_{max}$ values of HBUP formation were estimated from the fitted data. Determination of appropriateness of fit to each kinetic model was accomplished by examination of the sum of squares of residuals, the size of the coefficients of variations and standard errors of the parameter estimates.
Results

Correlation between BUP hydroxylase and BROD activities in RHM

Correlations between BUP hydroxylase and BROD activities were examined in RHMs isolated from various rat hepatocyte cultures, controls (n=5) or treated with CYP2B1 inducer (PB, n=3), CYP3A1 inducer (DEX, n=5), CYP4A inducer (FEN, n=4) or CYP1A2 inducers (3-MC or BNF, n=5) (Fig. 2). The substrates were used in excess, i.e., at a concentration of 500 µM BUP and 20.5 µM benzyloxyresorufin. When including 3-MC and BNF-treated groups, BUP hydroxylase activity did not correlate well with BROD activity ($r^2 = 0.49$, $p = 0.014$, Fig. 2; ■). A notable difference between BROD and BUP hydroxylase activities was with respect to CYP1A2 inducer (3-MC and BNF) treatment. Both compounds induced BROD activity but did not induce BUP hydroxylase activity. In contrast, BUP hydroxylase activity from controls, DEX-, FEN- and PB-treated group (4.17–53.72 pmol/min/mg protein) significantly correlated with BROD (0.15–27.87 pmol/min/mg protein; $r^2 = 0.81$, $p < 0.01$; Fig. 2; ◆). This suggests that BROD was not selective for CYP2B1 activity, since neither 3-MC nor BNF would be expected to induce CYP2B1.

Inhibition Experiments

The pilot experiment was performed to confirm the ability of proadifen and metyrapone to inhibit the BUP hydroxylation and BROD by using cDNA-expressed CYP2B1. Proadifen and metyrapone effectively inhibited cDNA-expressed CYP2B1-dependent BUP hydroxylase activity with an IC$_{50}$ of 14.9 ± 3.7 and 7.52 ± 0.54 µM, respectively (Table 1). These two inhibitors also effectively inhibited cDNA-expressed CYP2B1-dependent BROD activity with an IC$_{50}$ of 38.1 ± 1.3 and 1.85 ± 1.11 µM, respectively (Table 1). To determine the potential substrate selectivity of BUP for CYP2B1 but not CYP1A2 involved in these reactions, liver microsomes from BNF- and PB-pretreated rats were used. BROD activity and BUP hydroxylation were measured in these microsomes pre-incubated or not with three known CYP inhibitors in the presence of substrate prior to the addition of NADPH which initiates enzyme activity (Table 1). When incubated with PB-induced RLM, proadifen (CYP1A2/2B1 inhibitor) and metyrapone (CYP2B1 inhibitor) caused a great reduction in both BROD and BUP hydroxylation, the IC$_{50}$ being 1.21 ± 0.49 and 3.89 ± 1.54 µM for BUP hydroxylation and 0.688 ± 0.161 and 0.806 ± 0.663 µM for BROD, respectively as seen in Table1. By contrast, in BNF-induced RLM, the IC$_{50}$ for BROD by ANF (CYP1A2 inhibitor) and proadifen was much lower (2.5x10$^{-3}$ ± 0.8x10$^{-3}$ µM) than that of BUP hydroxylation (>100 µM), while the IC$_{50}$ for BROD activity and BUP hydroxylation were equivalent (40.8 ± 4.6 and 41.8 ± 3.4 µM, respectively) in the presence of metyrapone (CYP2B1 inhibitor).
Taken all together, these results suggest that CYP2B1 is similarly involved in both activities, whereas CYP1A1/2 is involved in BROD activity, but not in BUP hydroxylation.

**Evaluation of BUP Hydroxylation by Individual cDNA-expressed Rat CYPs.**

A panel of fourteen cDNA-expressed enzymes (SUPERSOMES) was screened for BUP hydroxylase activity at 500 and 2500 µM BUP (Fig. 3). These substrate concentrations were selected to ensure saturation of any high Km isozyme capable of catalyzing BUP hydroxylation. cDNA-expressed CYP2B1 exhibited the highest percentage of total BUP hydroxylation at 2500 µM BUP (75%), as compared to 63% at 500 µM BUP. Two other cDNA-expressed CYPs also contributed to BUP hydroxylation but to a lower rates: CYP2C11 participated for 23% and 8.7% of total HBUP, at 500 and 2500 µM BUP respectively, and CYP2E1 participated for 1.8 and 10.9% of total HBUP, respectively at 500 and 2500 µM BUP. Rates of BUP hydroxylation by CYP1A1, CYP1A2, CYP2A1, CYP2A2, CYP2C6, CYP2C12, CYP2C13, CYP2D1, CYP2D2, CYP3A1 and CYP3A2 were less than 10% of total HBUP formation. HBUP formation in control microsomes was undetectable.

**Kinetic Analyses of BUP Hydroxylation**

Kinetic parameters of BUP hydroxylation were first estimated by fitting kinetic data with PB-pretreated RLM (Fig. 4A) and by fitting kinetic data with cDNA-expressed CYP2B1 (Fig. 4B) to the single enzyme Michaelis-Menten equation. The apparent $K_m$ and $V_{max}$ for BUP hydroxylation in the PB-pretreated RLM with single enzyme kinetics were $158.5 \pm 23.1$ µM and $1697 \pm 81$ pmol/min/mg protein, respectively (Table 2) and the apparent $K_m$ and $V_{max}$ for BUP hydroxylation by cDNA-expressed CYP2B1 were $152.5 \pm 11.5$ µM and $181.2 \pm 5.5$ pmol/min/pmol CYP, respectively (Table 2). Kinetic parameters of BUP hydroxylation were also estimated by fitting kinetic data with cDNA-expressed CYP 2E1, 2C6, 2C11 and 3A1. The apparent $K_m$ and $V_{max}$ for BUP hydroxylation by cDNA-expressed CYP2E1 were $914 \pm 305$ µM and $0.143 \pm 0.020$ pmol/min/pmol CYP, respectively (Fig. 5A). Substrate inhibition for BUP hydroxylation was observed with cDNA-expressed CYP2C6 (Fig. 5B), 2C11 (Fig. 5C) and 3A1 (Fig. 5D) for BUP concentrations over 500 µM. Using the substrate inhibition model (Lin et al., 2001), the $K_m$ for BUP hydroxylation by cDNA-expressed CYP2C6, 2C11 and 3A1 were $67.8 \pm 11.8$, $190 \pm 52.5$ and $453 \pm 81.7$ µM, respectively. $V_{max}$ for BUP hydroxylation by cDNA-expressed CYP2C6, 2C11 and 3A1 were $0.219 \pm 0.016$, $1.65 \pm 0.30$ and $0.376 \pm 0.044$ pmol/min/pmol CYP, *i.e.* more than 100 times lower than that of cDNA-expressed CYP2B1.
Discussion

Over the past several decades, CYP2B enzymes have served as prototypical models for investigation of the mechanism by which drugs and environmental contaminants activate gene expression. CYP2B enzymes are also very versatile catalysts with a broad range of substrates including drugs, environmental pollutants, and steroids (Kedzie et al., 1991). CYP2B is the main hepatic CYP isoform inducible by PB and other barbiturates in experimental animals, CYP2B-mediated biotransformations being extensively studied in both small rodents and in rabbit (Nims and Lubets, 1996). In humans, BUP hydroxylation has been shown to be a selective marker of CYP2B6 (Faucette et al., 2000) but to our knowledge, a fully selective probe for CYP2B1 catalytic activity in rodents, that would facilitate further examination of the role of this enzyme in xenobiotic metabolism, has not been described. Indeed although BROD is routinely used for evaluating CYP2B1 activity (Burke et al., 1985, 1994; Lubet et al., 1985), it can also be partly related to CYP1A2 activity (Kobayashi et al., 2002; Chovan et al., 2007). In a previous study (Richert et al., 2009), we found a good correlation between BUP hydroxylation and CYP2B1 mRNA expression in rat hepatocyte cultures. The results of the present study support the use of BUP at saturating concentrations as a selective in vitro probe substrate for the determination of CYP2B1 catalytic activity.

Previous reports described that BROD reaction clearly involved multiple enzymes: according to Kobayashi et al. (2002), CYP2B1 accounted for 60% and CYP1A2 for 35% and according to Chovan et al. (2007) the respective involvements of CYP2B1 and CYP1A2 were 32% and 34%. In the present study, the apparent $K_m$ for BUP hydroxylation in PB-pretreated RLM (158.5 ± 23.1 µM) was equivalent to that of cDNA-expressed CYP2B1 (152.5 ± 11.5 µM). Although this observation alone cannot support the conclusion that a single enzyme is involved in BUP hydroxylation in RLM, these results are in accordance with data obtained with human CYP2B6 for which it has been reported that BUP is metabolized primarily by CYP2B6 to HBUP with an apparent $K_m$ of 107.5 ± 20.5 µM (Ekins et al., 1999) or of 155.8 ± 18.2 µM (Faucette et al., 2000) in human cDNA-expressed CYP2B6 and an apparent $K_m$ of 130.2 ± 22.0 µM in human liver microsomes (Faucette et al., 2000).

The results from inhibition experiments by using IC$_{50}$ values as a measure of the efficacy of inhibition of microsomal CYP1A2 and CYP2B1 activities, further suggest the selectivity of CYP2B1 for BUP hydroxylation but not BROD. Proadifen and metyrapone effectively inhibited cDNA-expressed CYP2B1-dependent BUP hydroxylase activity with an IC$_{50}$ of 14.9 ± 3.7 and 7.52 ± 0.54 µM, respectively. IC$_{50}$ for BROD and BUP hydroxylation were equivalent (40.8 ± 4.6 and 41.8 ± 3.4 µM, respectively) when using liver
microsomes from BNF-pretreated rats in the presence of metyrapone, a CYP2B1-selective inhibitor. However, when using liver microsomes from rats pretreated with BNF, in the presence of ANF a CYP1A1/2-selective inhibitor, we found an IC$_{50}$ of 2.5x10$^{-3} \pm 0.8x10^{-3}$ µM for BROD while >100 µM for BUP hydroxylation. These results suggest that CYP2B1 is similarly involved in both activities, whereas CYP1A1/2 is involved in BROD activity, but not in BUP hydroxylation. This is further supported by the good correlation of BUP hydroxylation with BROD activity ($r^2 = 0.81, p < 0.01$; Fig. 2) when considering control, DEX-, FEN- and PB-treated rat hepatocytes cultures but poor correlation when including rat hepatocytes treated with CYP1A2 inducer (3-MC and BNF) ($r^2 = 0.49, p = 0.014$). In CYP1A2 induced rat hepatocytes, BROD activity was increased while BUP hydroxylase activity was unchanged, clearly demonstrating that BROD is not selective for CYP2B1 activity, since neither 3-MC nor BNF would be expected to induce CYP2B1. Our data confirm the principle role of CYP2B1 in catalyzing HBUP formation, as suggested by our finding of a good correlation between CYP2B1 expression and BUP hydroxylation but not BROD activity (Richert et al., 2009).

Since the early 1990s, the use of cDNA-expressed CYPs has assisted, in addition to liver microsomes, in the evaluation of metabolic specificity of probe substrates and the identification of the CYP enzymes involved in the metabolism of xenobiotics in human and rat liver microsomes. We show that, among a panel of fourteen rat cDNA-expressed CYP isozymes (CYP1A1, CYP1A2, CYP2A1, CYP2A2, CYP2B1, CYP2C6, CYP2C11, CYP2C12, CYP2C13, CYP2D1, CYP2D2, CYP2E1, CYP3A1 and CYP3A2), the rate of BUP hydroxylation at high concentrations of BUP (500 µM and 2500 µM) was highest with CYP2B1. When using BUP at 2500 µM to ensure saturation of any high $K_m$ isozyme capable of catalyzing BUP hydroxylation, two other CYP isozymes, i.e. CYP2C11 and CYP2E1, were also found to be catalytically competent (8.7% and 10.9% of total HBUP formation respectively, as compared to 75% for CYP2B1). Previous in vitro studies dealing with the involvement of human CYP using various BUP concentrations reached similar conclusions (Chen et al., 2010); also Faucette et al. (2000, 2001) and Hesse et al. (2000) both reported that although CYP2B6 was the major isoform (71%) involved in BUP hydroxylation, CYP2E1 and CYP3A4 can also be involved in BUP hydroxylation (respectively 24% and 2%).

Kinetic parameters of BUP hydroxylation were estimated by fitting kinetic data with cDNA-expressed CYP2C6, 2C11, 2E1 and 3A1. Kinetic data for cDNA-expressed CYP2E1 ($K_m$ of 914 ± 305 µM and $V_{max}$ of 0.143 ± 0.02 pmol/min/pmol CYP) and for cDNA-expressed CYP3A1 ($K_m$ of 453 ± 51 µM and $V_{max}$ of 0.376 ± 0.044 pmol/min/pmol CYP) reveal that these isoforms are not involved in BUP hydroxylation. By contrast, $K_m$ values for BUP hydroxylation with cDNA-expressed CYP2C6 (67.8 ± 11.8 µM) and CY2C11...
(190 ± 52.5 µM), were close to that of CYP2B1 (152.5 ± 11.5 µM) and suggest that at low concentrations of BUP, the CYP2C isoforms can be involved in BUP metabolism. As plasmatic concentrations of BUP in rat have been shown to be 4 µM (Suckow et al., 1986), BUP cannot be used in vivo as a marker for CYP2B1 activity. However, the apparent \( V_{\text{max}} \) for CYP2C isoforms were very low (0.22 ± 0.02 pmol/min/pmol CYP for CYP2C6 and 1.65 ± 0.30 pmol/min/pmol CYP for CYP2C11) compared to \( V_{\text{max}} \) for BUP hydroxylation with cDNA-expressed CYP2B1 (181.2 ± 5.5 pmol/min/pmol CYP). In addition, substrate inhibition of the CYP2C isoforms occurs from BUP concentrations over 500 µM, while no such effect was found with CYP2B1. As a consequence, the involvement of the CYP2C isoforms in BUP hydroxylation at saturating concentrations of BUP are negligible compared to CYP2B1.

Taken all together, the data suggest that BUP is a selective probe substrate for CYP2B1, characterized by a relatively high \( K_m \) value (158.5 µM) that maintains CYP selectivity due to the absence of other significantly contributing CYP isozymes, especially at saturating BUP concentrations. Other selective substrates have high \( K_m \) value such as the caffeine for human CYP1A2 (\( K_m \) ~ 1.2 mM) (Hickman et al., 1998), tolbutamide for human CYP2C9 (\( K_m \) ~ 200 µM) (Hickman et al., 1998) and testosterone for rat CYP3A1 (\( K_m \) ~ 150 µM) (Cooper et al., 1993).

In conclusion, this study validates BUP hydroxylation as an in vitro diagnostic marker for CYP2B1 catalytic activity when assayed at >500 µM BUP. This will now allow the assessment of the contribution of CYP2B1 to the metabolism of the given drug.
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Authorship contributions

Participated in research design: Pekthong, Richert, Martin

Conducted experiments: Pekthong, Desbans

Performed data analysis: Pekthong, Richert, Martin

Wrote or contributed to the writing of the manuscript: Pekthong, Richert, Martin
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Footnotes

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Legends for figures

Figure 1. Structure of bupropion and hydroxybupropion (major active metabolite)

Figure 2. Correlation between BUP hydroxylase and BROD activities in microsomes from rat hepatocytes, control and treated with CYP2B, CYP3A1 and CYP4A inducers (n = 17; ◆) or with CYP1A2 inducers (n=5; ■). BUP hydroxylase activity was determined by incubating 500 µM BUP, 0.05 mg of microsomal protein, 62.5 mM KH₂PO₄ (pH 7.4), 1 mM EDTA, 3 mM MgCl₂, and 1 mM NADPH for 30 min. HBUP formation was quantitated by HPLC and normalized for microsomal protein and incubation time. BROD activity was determined by incubating with 20.5 µM benzyloxyresorufin. Resorufin formation was quantified by spectrophotometry. The correlation of the two activities was evaluated by linear regression analysis. Statistical significance was assessed by an F test.

Figure 3. Rates of HBUP formation were assessed in microsomes from baculovirus-infected insect cells (SUPERSOMES) that expressed cDNA for NADPH-P450 oxidoreductase and CYP1A1, CYP1A2, CYP2A1, CYP2A2, CYP2B1, CYP2C6, CYP2C11, CYP2C12, CYP2C13, CYP2D1, CYP2D2, CYP2E1, CYP3A1 or CYP3A2. HBUP formation was quantitated by LC/MS/MS and normalized for pmol of CYP and incubation time. BUP concentration, CYP amount, and incubation time were 500 and 2500 µM, 50 pmol, and 30 min, respectively. Control incubations were also conducted with insect cell-derived microsomes transfected with baculovirus only. Data are expressed as % of total HBUP formation.

Figure 4. Michaelis-Menten plots of HBUP after the incubation of BUP (concentration range 25 to 2500 µM) with PB-induced RLMs (A) or microsomes containing cDNA-expressed CYP2B1 (B); each value is a mean ± S.E.M. of triplicate determinations.

Figure 5. Michaelis-Menten plot of HBUP after the incubation of BUP (concentration range 25 to 2500 µM) with microsomes containing cDNA-expressed CYP2E1 (A) and Substrate-inhibition plot of HBUP after the incubation of BUP (concentration range 25 to 2500 µM) with microsomes containing cDNA-expressed CYP2C6 (B), microsomes containing cDNA-expressed CYP2C11 (C) and microsomes containing cDNA-expressed CYP3A1 (D); each value is a mean ± S.E.M. of triplicate determinations.
Tables

**Table 1.** Inhibition of BROD and BUP hydroxylase activities in β-naphthoflavone (BNF)-, phenobarbital (PB)-pretreated RLM and c-DNA expressed CYP2B1 by α-naphthoflavone (ANF), proadifen and metyrapone. BUP hydroxylase activity was determined by incubating 500 μM BUP, 0.05 mg of microsomal protein, and 1 mM NADPH for 30 min. HBUP formation was quantitated by HPLC. BROD activity was determined by incubating with 20.5 μM benzyloxyresorufin and 1 mM NADPH for 2 min. Resorufin formation was quantitated by spectrofluorometry. IC$_{50}$ values are the mean ± S.E.

<table>
<thead>
<tr>
<th>Reactions</th>
<th>Liver microsomes</th>
<th>Inhibitory effects; IC$_{50}$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ANF</td>
</tr>
<tr>
<td>BROD</td>
<td>BNF-pretreated</td>
<td>0.00248±0.00084</td>
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<tr>
<td></td>
<td>PB-pretreated</td>
<td>&gt;100</td>
</tr>
<tr>
<td></td>
<td>c-DNA expressed</td>
<td>ND$^a$</td>
</tr>
<tr>
<td></td>
<td>CYP2B1</td>
<td></td>
</tr>
<tr>
<td>BUP hydroxylation</td>
<td>BNF-pretreated</td>
<td>&gt;100</td>
</tr>
<tr>
<td></td>
<td>PB-pretreated</td>
<td>&gt;100</td>
</tr>
<tr>
<td></td>
<td>c-DNA expressed</td>
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</tr>
<tr>
<td></td>
<td>CYP2B1</td>
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</tbody>
</table>

$^a$ND = not determined
Table 2. Kinetic parameters of BUP hydroxylation in PB-treated rat liver microsome (RLM) and in microsome containing cDNA-expressed CYP2B1.

Rates of HBUP formation were determined in triplicate at 25 to 2500 μM BUP in RLMs and in CYP2B1-containing insect cell-derived microsomes. HBUP formation was quantitated by LC/MS/MS. Weighted concentration-rate data were fit to a one-component Michaelis-Menten model by nonlinear regression using the GraphPad program. Kinetic parameters were estimated from the fitted data.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Model equation</th>
<th>(K_m^a)</th>
<th>(V_{max}^a)</th>
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<tbody>
<tr>
<td>RLM</td>
<td>Michaelis-Menten</td>
<td>158.5±23.1 μM</td>
<td>1697±81 pmol/min/mg protein</td>
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<tr>
<td>cDNA-expressed CYP2B1</td>
<td></td>
<td>152.5±11.5 μM</td>
<td>181.2±5.5 pmol/min/pmol CYP</td>
</tr>
</tbody>
</table>

\(^a\)Parameter estimated ± S.E.M.
Figure 1

Bupropion

Hydroxybupropion
Figure 2

![Graph showing the relationship between BROD activity (pmol/min/mg protein) and Bupropion hydroxylase activity (pmol/min/mg protein)].

- $R^2 = 0.81$
- $p < 0.01$
Figure 3

The graph shows the percentage of total HBUP formation for various cDNA-expressed enzymes under different concentrations of BUP (2500 µM and 500 µM). The y-axis represents the percentage of total HBUP formation, while the x-axis lists the cDNA-expressed enzymes. The black bars indicate the 2500 µM BUP treatment, and the gray bars indicate the 500 µM BUP treatment.
Figure 4

(A) pmol/min/mg protein vs. [Bupropion] (μM)

(B) pmol/min/pmol CYP vs. [Bupropion] (μM)