Deeper Insight into the Reducing Biotransformation of Bupropion in the Human Liver

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Summary: Bupropion is widely used as an antidepressant drug and also as a smoking cessation aid. In humans, this drug is extensively metabolized to form several metabolites. Oxidised hydroxybupropion and two reduced metabolites, threohydrobupropion and erythrohydrobupropion, are major metabolites. All of these metabolites are considered to be active. Although the oxidative metabolic pathway and the central role of CYP2B6 are known, the enzymes that participate in the reduction have not been identified to date. The aim of this study was to confirm the role of human liver subcellular fractions in the metabolism of bupropion and elucidate the contribution of particular carbonyl-reducing enzymes. An HPLC method for the determination of bupropion metabolites was utilised. Bupropion is reduced to threohydrobupropion and less to erythrohydrobupropion in human liver cytosol, microsomes and also mitochondria. Surprisingly, intrinsic clearance for formation of both metabolites is the highest in mitochondrial fraction. Moreover this study provides the first direct evidence that 11β-hydroxysteroid dehydrogenase 1, AKR1C1, AKR1C2, AKR1C3 and CBR1 participate in the reducing biotransformation of bupropion in vitro. The enzyme kinetics of all of these reductases was investigated and kinetic parameters were calculated.

Keywords: biotransformation; bupropion; carbonyl; metabolism; reductases; AKR; SDR; enzyme kinetics

Introduction

Bupropion (BUP) was introduced clinically in 1985 as an antidepressant agent and currently is widely prescribed. BUP is also used as a smoking cessation aid and is currently being tested as a candidate for the treatment of drug abuse, obesity, and eating disorders.1,2) The exact mechanism through which BUP acts has not yet been elucidated, although it most likely includes a combination of several diverse possibilities, such as the inhibition of the dopamine and norepinephrine transporters and the blockade of the acetylcholine receptor.3)

After its administration, BUP is almost completely absorbed and extensively metabolised. Less than 1% of the administered BUP is eliminated in the urine or faeces as the parent drug. The human phase I biotransformation of BUP leads to the formation of three major metabolites: hydroxybupropion (HB), threohydrobupropion (TB) and erythrohydrobupropion (EB) (Fig. 1) and some minor metabolites (e.g. m-chlorobenzoic acid). These metabolites are subsequently conjugated with glucuronic acid during a phase II biotransformation and excreted through the urine.4–6)

The three major phase I metabolites are regarded as active antidepressants: HB is half as potent as BUP, the activity of TB reaches 20 to 50% of the activity of BUP, and EB possesses approximately 20% of the activity of BUP.2,7) Moreover, the concentration of the metabolites in the plasma is several-fold higher than the concentration of the BUP8–10) and the elimination of TB and EB is slower in comparison with the parent drug.2,7) As is mentioned above, at least part of the antidepressant activity of BUP is attributable to the metabolites so these may also contribute to the effect of BUP as a smoking cessation aid.11–13) Because all of the...
above-mentioned phase I metabolites are active, it is important to know which enzymes are responsible for the formation of the metabolites that participate in the pharmacological action of BUP. The oxidative biotransformation of most drugs is commonly well known, and BUP is not an exception. The main enzyme responsible for the formation of the oxidative metabolite HB is CYP2B6,\textsuperscript{14,15} and other isoforms play a minor role in the formation of HB\textsuperscript{16} or participate in the metabolism of BUP to form minor metabolites.\textsuperscript{17} A completely different situation exists in the case of the formation of the reduced metabolites TB and EB in humans. Knowledge about the reductive biotransformation of BUP is restricted only to placental and liver subcellular fractions, and the identification of the participating carbonyl-reducing enzymes has only been performed indirectly through inhibition studies. Two studies mentioned 11$\beta$-hydroxysteroid dehydrogenases (11$\beta$-HSDs) and carbonyl reductases (CBRs) as enzymes that participate in the formation of TB and EB.\textsuperscript{7,18} However, the group of carbonyl-reducing enzymes is quite large and involves two superfamilies: aldo-keto reductases (AKR) and short-chain dehydrogenases/reductases (SDR). Together, these superfamilies contain nearly 100 described human enzymes.\textsuperscript{19,20} Although EB is generally formed to a lesser extent, the amount of TB produced is approximately in the same order as the amount of HB.\textsuperscript{8} The TB concentration is sometimes even higher than that of HB (e.g., in the cerebrospinal fluid\textsuperscript{21} and in the plasma after overdose or abuse\textsuperscript{11,22}), and this fact supports the importance of the identification of the enzymes responsible for its production.

The aim of this study was to elucidate the role of particular well-known human biotransformation carbonyl-reducing enzymes in the metabolism of BUP and to compare the formation of the reduced metabolites TB and EB with that of the oxidative metabolite HB in human liver subcellular fractions. Ten human carbonyl-reducing enzymes were prepared in their recombinant forms, and a modified HPLC method was developed for the determination of BUP and its major metabolites. This study provides the first demonstration that five human carbonyl-reducing enzymes (three from the AKR superfamily and two from the SDR superfamily) participate in the metabolism of BUP in vitro.

Methods

Chemicals: BUP hydrochloride, HB, racemate EB, and racemate TB were purchased from Toronto Research Chemical (Toronto, Canada). The mobile phase consists of HPLC-grade acetonitrile, triethylamine, sodium phosphate monobasic (Sigma-Aldrich, Prague, Czech Republic), and HPLC-grade water, which was prepared using the Millipore Milli Q reverse osmosis Millipore system (Millipore, Bedford, MA) and filtered through a vacuum filter (Vac-space 50 vacuum filter, Chromservis, Prague, Czech Republic). The NADPH-generating system was composed of NADPH, glucose-6-phosphate dehydrogenase, magnesium chloride (Sigma-Aldrich), and glucose-6-phosphate dehydrogenase (Roche, Basel, Switzerland). All of the other chemicals were of the highest purity that was commercially available.

For the cloning and preparation of the recombinant forms of the carbonyl-reducing enzymes, the following reagents were used: full-length cDNA coding sequences (Source Bioscience, Cambridge, UK), primers (Generi Biotech, Hradec Kralove, Czech Republic), Phusion Start II High Fidelity DNA Polymerase (Finnzymes, Vantaa, Finland), T4 DNA ligase (New England BioLabs, Ipswich, MA), restriction endonucleases (New England BioLabs), the expression vector pET-28b(+), and a Bug Buster Protein Extraction system (Novagen-Merck, Darmstadt, Germany). For the preparation of the recombinant form of 11$\beta$-HSD1, the following were used: the full-length cDNA (Structural Genomics Consortium, Oxford, UK), Platinum PfX DNA polymerase (Invitrogen, Carlsbad, CA), T4 DNA polymerase (Fermentas, Vilnius, Lithuania), restriction enzymes (New England Biolabs), a baculovirus expression system (Bac-to-Bac System; Introvigen), a Nanofectin kit (PAK, Pasching, Austria), and S9 insect cells (Spodoptera frugiperda).

Preparation of human liver subcellular fractions: The human liver samples were obtained from the Cadaver Donor Programme of the Transplant Centre of the Faculty of Medicine (Hradec Kralove, Czech Republic) in accordance with Czech legislation. These tissue samples were processed to obtain subcellular fractions as described previously.\textsuperscript{23}

Preparation of the recombinant forms of cytosolic carbonyl-reducing enzymes: The preparation of recombinant CBR1, CBR3, AKR1A1, AKR1B1, AKR1B10, AKR1C1, AKR1C2, AKR1C3, and AKR1C4 was performed according to the standard techniques in the Escherichia coli expression system described in the laboratory manual published by Sambrook.\textsuperscript{24} In brief, the coding sequence of each enzyme was amplified from a human liver cDNA library by PCR using primers that were 18 to 21 nucleotides in length (Table 1).

The PCR fragments were purified and ligated into the pET-28b(+) vector using NdeI/Xhol restriction sites and transformed into the calcium chloride–competent E. coli strain HB101 using the heat shock method. Amplified vectors with the coding sequence for a particular enzyme were sequenced (Generi Biotech,) to confirm the absence of mutation. All of the prepared constructs were transformed into the calcium chloride–competent E. coli strain BL21 (DE3) using the heat shock method. For the expression of a particular protein, 200 ml of LB medium containing 50 mg/ml kanamycin was inoculated with BL21(DE3) cells transformed with the appropriate recombinant plasmid. The cells were allowed to grow at 37°C until an OD$_{600}$ of 0.6 was achieved. Isopropyl $\beta$-D-1-thiogalactopyranoside was added to a final concentration of 1 mM, and the culture was grown for an additional 4–5 h. The cells that overexpressed the target protein were pelleted by centrifugation at 4°C and resuspended in the BugBuster Protein Extraction reagent. The supernatants containing the overexpressed His-tagged proteins were purified by Ni-affinity chromatography with the Äkta purifier system and a HisTrap 1 ml column (GE Healthcare, Stockholm, Sweden). Then, 20 mM Tris buffer, pH 7.8, containing 30 mM imidazole, 500 mM NaCl, and 10% (v/v) glycerol was used for the
purification of the proteins, and an increasing gradient of imidazole from 30 to 500 mM was used for the elution of the proteins. The successful overexpression and purification of a particular enzyme was monitored by SDS-PAGE. The pure active enzymes were stored in 20 mM Na-phosphate buffer, pH 7.4, with 20–40% glycerol. The protein concentration was determined using a BCA Protein Assay kit.

**Preparation of microsomal 11β-hydroxysteroid dehydrogenase 1:** The recombinant form of human 11β-HSD1 was prepared by taking advantage of the baculovirus expression system. Briefly, the cDNA of 11β-HSD1 was amplified by PCR (specific forward primer 5'-TTAAGAGGAGATATACTATGAACTGGGAGCTG-3' and the reverse primer 5'-GATTTGGAATGAGTTTCTGCTGCTGTGTTTCTTTTAAAG-3'), which contained a sequence for ligation-independent cloning (LIC). The purified PCR product was subcloned into the pFB-CT10HF-LIC transfer vector (kindly provided by Structural Genomics Consortium), and the final construct was transformed into E. coli cells (XL1-blue supercompetent cells). The transformants were confirmed by PCR using sequencing primers (forward primer 5'-TTAAGAGGAGATATACTATGAACTGGGAGCTG-3' and reverse primer 5'-GATTTGGAATGAGTTTCTGCTGCTGTGTTTCTTTTAAAG-3'). The target construct was transformed into DH10Bac E. coli cells for transposition into the baculovirus shuttle vector (bacmid). The resulting bacmid was verified through blue/white selection and subsequently through sequencing of the purified bacmids from the positive colonies. The isolated bacmids were used to infect a suspension culture of Sf9 cells, and the infected cells according to the manufacturer's instructions (Nanofectin kit; PPA). The recombinant virus with 11β-HSD1 was amplified and used to infect a suspension of Sf9 cells (40 µg in each reaction), cytosol prepared human liver microsomes (40 µg in each reaction), and 0.1 M Na-phosphate buffer, pH 7.4, with 20–40% glycerol. The protein concentration was determined using a BCA Protein Assay kit.

**Optimisation of the extraction of the bupropion metabolites:** The stock solutions of HB, TB, and EB [1 mM in the 20:80 (v/v) mixture of acetonitrile and water] were dissolved and mixed with 0.1 M Na-phosphate buffer, pH 7.4, to a final concentration of 5 µM in a total volume of 100 µl. Then, 40 µl of 25% NH₃ and cooling to 0°C on an ice bath. The generated metabolites of BUP were extracted to 1,000 µl of ethyl acetate, chloroform, or hexane was performed for 1, 5, or 15 min. Repeated extractions under the same conditions were also performed but there was no significant improvement in the efficiency.

**Bupropion biotransformation in subcellular fractions:** The prepared human liver microsome (40 µg in each reaction), cytosol (80 µg in each reaction) or mitochondria (80 µg in each reaction) were incubated in 0.1 M Na-phosphate buffer, pH 7.4, with BUP at a final concentration of 100 µM in the presence of the NADPH generating system (final concentrations: 0.8 mM NADPH, 6 mM glucose-6-phosphate, 35 units glucose-6-phosphate dehydrogenase, and 3 mM MgCl₂) for 45 min at 37°C (final volume of the reaction mixture was 100 µl). The reactions were terminated by the addition of 40 µl of 25% (v/v) NH₃ and cooling to 0°C on an ice bath. The generated metabolites of BUP were extracted to 1,000 µl of ethyl acetate by shaking for 15 min. Each sample was centrifuged for 2 min at 13,000 rpm. The upper organic phase was transferred to a new Eppendorf microtube and evaporated to dryness under vacuum. The residues were dissolved in 100 µl of the mobile phase, and 60 µl was injected into the HPLC system. Control experiments without biological material were also performed to determine the non-enzymatic reduction of BUP.

**Bupropion reductase assay:** First, 4 µl of an individual pure recombinant carbonyl-reducing enzyme prepared according to the method described above and 20 µg of the Sf9 microsomal fraction with overexpressed 11β-HSD1 were incubated using the procedure prepared daily by dissolving the substances in a 20:80 (v/v) mixture of acetonitrile and water. The reference standard solution was prepared by diluting the stock solutions to 5 µM. Samples of 50 µl were injected into the chromatographic system and analyzed using an HPLC Agilent 1100 series (Agilent, Santa Clara, CA).

| Table 1. PCR primers used for the cloning of the carbonyl-reducing enzymes |
|---------------------------------|---------------------------------|
| **Forward primers** | **Reverse primers** |
| CBR1 5'-GGATCCATAATGTCTGCTGCGGATC-3' | 5'-GGCCTCGAGTCACTCTGCTCAACT-3' |
| CBR3 5'-GGATCCATAATGTCTGCTGCGGATC-3' | 5'-GGCCTCGAGTCACTCTGCTCAACT-3' |
| AKR1A1 5'-GCACCAACATGGGGCTCTCCTG-3' | 5'-CCGGTCTCGAGTCACTCTGCTCAACT-3' |
| AKR1B1 5'-CTGGGCAATAGGCGAGGCTG-3' | 5'-ACGGCTCGAGTCACTCTGCTCAACT-3' |
| AKR1B10 5'-CTTCCAACATAGGGCGAGGCTG-3' | 5'-ACGGCTCGAGTCACTCTGCTCAACT-3' |
| AKR1C1 5'-CTGGAACCATAGGCTGGATCAATATCATC-3' | 5'-GGGCGGCTCGAGTATTTATCATCAGAA-3' |
| AKR1C2 5'-CGTGCGGATAATAGGGTGTCGAGT-3' | 5'-GCCGATCTCTATAATCTGCTGTAAT-3' |
| AKR1C3 5'-GGATCCATAATGGTCTCCCAAAACACAG-3' | 5'-GCCGATCTCTATAATCTGCTGTAAT-3' |
| AKR1C4 5'-CGGATCCATAATGGTCTCCCAAAACACAG-3' | 5'-GCCGATCTCTATAATCTGCTGTAAT-3' |

The restriction sites for the NdeI and XhoI restriction enzymes are underlined.

**Development of analytical method:** Stock solutions of BUP, HB, TB, and EB at a concentration of 50 µM for each analyte were prepared daily by dissolving the substances in a 20:80 (v/v) mixture of acetonitrile and water. The reference standard solution was prepared by diluting the stock solutions to 5 µM. Samples of 50 µl were injected into the chromatographic system and analyzed using an HPLC Agilent 1100 series (Agilent, Santa Clara, CA).

The HPLC method optimisation was performed based on previously published methods. The chromatographic analysis was performed using a Waters Symmetry column (100 × 4.6 mm; 5 µm), and the mobile phase was composed of 20 mM sodium phosphate monobasic, pH 5.45 (the pH was adjusted using tri-ethylamine), and acetonitrile at a ratio of 80:20 (v/v). The flow rate was 1.2 ml/min at 45°C. The UV detection was performed at 214 nm.

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that was used for the subcellular fractions. If no metabolites were detected, the incubation was repeated with 8 µg of the enzyme and a higher concentration of BUP (final concentrations in the reaction mixture of 300 and 500 µM) to avoid any false negative results. Control experiments without biological material were also performed to determine the non-enzymatic reduction of BUP. The S9 microsomal fraction obtained after the infection of the S9 cells with wild-type (only sequence for green fluorescent protein) virus was used as a control for the evaluation of the 11β-HSD1 activity.

**Determination of enzyme kinetic parameters:** The subcellular fractions and the pure recombinant carbonyl-reducing enzymes that exhibited activity toward BUP were incubated using the methods described above. The final concentrations of BUP were in the range of 50 to 700 µM. The enzymatic activities for the formation of particular metabolites were expressed as the specific enzyme activity, which represents the picomoles of the metabolites formed per mg of protein in one minute. Control experiments without biological material were also performed to determine the non-enzymatic reduction of BUP. The kinetic constants (V_max and K_M) were determined by fitting rate measurement data using nonlinear least square fitting of a Michaelis-Menten hyperbola or allosteric sigmoidal kinetic model (GraphPad Prism 5.0 computer software). The values are presented as the mean ± S.D. of triplicates. CL_int represents enzymatic efficiency and is defined as V_max/K_M.

**Results**

**HPLC method and extraction of metabolites:** To study the phase I biotransformation of BUP, a modification of a published HPLC method was performed. Figure 2 shows representative chromatograms of the 250 nmol of HB (1), EB (2), TB (3), and BUP (4).

Additionally, an optimisation of the extraction of the metabolites from biological samples was performed as described in Methods section. The optimal recovery was obtained through the termination of the reaction with NH_3 and simple extraction into 1,000 µl of ethyl acetate; the extraction yields of HB, EB, and TB were 73 ± 1.1%, 76 ± 2.0%, and 60 ± 1.4%, respectively. A significant improvement in the yields was not achieved by multiple extractions (data not shown).

**Metabolism of BUP in human liver subcellular fractions:** The initial screening incubation of the subcellular fractions (cytosol, microsomes, and mitochondria) with 100 µM BUP showed that all three fractions participate in the formation of the reduced metabolites EB and TB, whereas HB is produced only in the microsomes and mitochondria. Surprisingly, TB is a major metabolite formed in all of the subcellular fractions. EB constitutes approximately 20% of the total metabolites formed in the cytosol and approximately 3% and 9% of the total metabolites in the microsomes and mitochondria, respectively. HB forms approximately 10% of the total metabolites in both the microsomes and the mitochondria.

A substrate range of 50 to 700 µM was used for the determination of the kinetic parameters in all of the subcellular fractions (Fig. 3). The kinetic parameters for the formation of all of the metabolites in the tested subcellular fractions are summarized in Table 2.

The human liver microsomes possess the lowest K_M for the formation of the reduced metabolite EB, whereas the lowest K_M for the formation of TB was found in the mitochondria. The metabolic potential of the tested fractions was compared, and it was found that the highest CL_int for the formation of both reduced metabolites was found in the mitochondrial fraction. Similar but slightly lower values were found in the microsomal fraction. In contrast, although the cytosol took part in the reduction of BUP, the efficiency of this reaction was many times lower compared with those found for the other tested fractions.

**Reduction of BUP by cytosolic carbonyl-reducing enzymes:** Four cytosolic enzymes take part in reduction of BUP in vitro: AKR1C1, AKR1C2, AKR1C3, and CBR1 (Fig. 4). None of the other tested enzymes displayed any activity toward BUP. All of the participating reductases exhibit different stereospecificity, which is characterized by a different ratio of the EB formed to the TB formed (Fig. 4). Through incubations with 100 µM BUP, activity of AKR1C1 in the production of EB is approximately 2.6-fold lower in comparison with TB, whereas activity of AKR1C3 in the same reaction is 10-fold lower compared to TB and CBR1 forms no EB. The production of EB by CBR1 is minor and able to be detected only at higher concentrations of BUP (≥300 µM); under such conditions activity in EB
formation is also roughly 10 times lower in comparison with TB formation. The kinetics of all enzymes was studied in the substrate range of 50 to 700 µM (Fig. 5), and all of the corresponding kinetic parameters were determined (Table 3). The Michaelis-Menten model of kinetics was observed for the formation of both EB and TB catalyzed by the AKR enzymes; CBR1 displays cooperativity and a better fit to the allosteric sigmoidal kinetic model with Hill constants of 2 and 3 for the formation of TB and EB, respectively.

The lowest values of \( K_M \) for the formation of both reduced metabolites were found for the reaction catalyzed by CBR1, but the \( V_{\text{max}} \) in both cases was very low. The highest \( V_{\text{max}} \) for the generation of EB and TB was observed in the reaction catalysed by AKR1C1. Moreover, AKR1C1 also possessed the highest \( CL_{\text{int}} \) and thus exhibits the highest efficiency in the cytosolic reduction metabolism of BUP in vitro.

**Reduction of BUP by microsomal carbonyl-reducing enzyme:** Only one microsomal carbonyl-reducing enzyme, 11\( \beta \)-hydroxysteroid dehydrogenase 1 (11\( \beta \)-HSD1), was tested to determine its participation in the biotransformation of BUP. The screening with 100 µM BUP showed that 11\( \beta \)-HSD1 is very active in the production of EB and TB compared with cytosolic enzymes (Fig. 4). EB is a minor metabolite formed by 11\( \beta \)-HSD1; activity of EB formation is roughly 20 times lower compared with activity of TB formation. Similarly to cytosolic carbonyl-reducing enzymes, the kinetic parameters were determined based on the Michaelis-Menten model (Fig. 5, Table 3). The \( K_M \) values for the formation of both reduced metabolites are lower compared with cytosolic enzymes, but the elimination potential represented by the value of \( CL_{\text{int}} \) for the formation of TB is approximately 8-fold higher than the \( CL_{\text{int}} \) of AKR1C1.

**Discussion**

Carbonyl reduction is an important phase I biotransformation reaction that often leads to the deactivation of drugs (e.g., anthracyclines) and sometimes to the formation of active metabolites (e.g., naltrexone and dolasetron). In some cases, the enzymes responsible for such reactions are well characterised (at least in vitro), e.g., the doxorubicin metabolism. However, most of the information on these reductases is either poor or absent, such as in the case of oxcarbazepine. The reducing phase I biotransformation of BUP has not yet been elucidated in detail. The oxidised metabolite HB is considered the main metabolite in vivo, but the discovery that TB may reach high values in plasma or other body fluids in vitro and in vivo is important for its identification. The reduction potential represented by the value of \( CL_{\text{int}} \) for the formation of TB is approximately 8-fold higher than the \( CL_{\text{int}} \) of AKR1C1.

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Fig. 5. Kinetics of the bupropion reduction to erythrohydrobupropion (EB) and threohydrobupropion (TB) catalysed by the cytosolic human recombinant enzymes AKR1C1, AKR1C2, AKR1C3, and CBR1 and the microsomal 11β-hydroxysteroid dehydrogenase 1 (11β-HSD1).

The Michaelis-Menten model of kinetics was used for the evaluation of the AKRs and the 11β-HSD1 enzymes, whereas the sigmoidal-allosteric model of kinetics better fits the results obtained with CBR1. The Eadie-Hofstee plot of the formation of EB and TB by 11β-HSD1 demonstrates a single enzyme system. The values are presented as the mean ± S.D. of 3 experiments.

### Table 3. Kinetic parameters of the reduction of BUP by cytosolic and microsomal human recombinant carbonyl-reducing enzymes

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Metabolite</th>
<th>K_M (µM)</th>
<th>V_max (pmol/mg protein/min)</th>
<th>CL_int (µl/mg protein/min)</th>
</tr>
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<tbody>
<tr>
<td><strong>Cytosol</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>AKR1C1</td>
<td>EB</td>
<td>584 ± 159</td>
<td>2,125.0 ± 318.3</td>
<td>3.64</td>
</tr>
<tr>
<td></td>
<td>TB</td>
<td>476 ± 87</td>
<td>5,073.0 ± 475.2</td>
<td>10.67</td>
</tr>
<tr>
<td>AKR1C2</td>
<td>EB</td>
<td>147 ± 61</td>
<td>13.3 ± 1.5</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>TB</td>
<td>460 ± 66</td>
<td>814.0 ± 59.0</td>
<td>1.76</td>
</tr>
<tr>
<td>AKR1C3</td>
<td>EB</td>
<td>981 ± 434</td>
<td>63.8 ± 19.2</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>TB</td>
<td>1,730 ± 604</td>
<td>5,030.0 ± 1,357.0</td>
<td>2.91</td>
</tr>
<tr>
<td>CBR1</td>
<td>EB</td>
<td>221 ± 21</td>
<td>8.9 ± 2.2</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>TB</td>
<td>316 ± 11</td>
<td>160.2 ± 18.4</td>
<td>0.51</td>
</tr>
<tr>
<td><strong>Endoplasmic reticulum</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11β-HSD1</td>
<td>EB</td>
<td>123 ± 30</td>
<td>349.4 ± 23.9</td>
<td>2.83</td>
</tr>
<tr>
<td></td>
<td>TB</td>
<td>76 ± 15</td>
<td>6,588.0 ± 283.1</td>
<td>86.40</td>
</tr>
</tbody>
</table>

The other tested cytosolic enzymes (AKR1B1, AKR1B10, AKR1C4, and CBR3) do not participate in the biotransformation of BUP.

*Allosteric-sigmoidal kinetic model; the Hill coefficients for the formation of EB and TB are n = 3 and n = 2, respectively.
cytosolic fraction exhibits the lowest activity for the reduction of BUP. Moreover, the human liver cytosol does not participate in the production of HB. The exact values of the kinetic parameters for the formation of EB and TB in hepatic subcellular fractions obtained in our study and the study performed by Molnari and Myers\textsuperscript{7} are quite distinct although in the same order of magnitude. The biggest differences are in \( V_{\text{max}} \) values (approximately 15-fold and 7-fold higher for formation of TB and EB in the microsomal fraction in the study performed by Molnari and Myers\textsuperscript{7}). There are several factors that may generally influence the activities of samples, e.g. the procedure for preparation of subcellular fractions, the age of organs and also interindividual differences in microsomal enzymes that have been found, e.g. in the human lung.\textsuperscript{30} In the above mentioned study, only the cytosolic and the microsomal fractions were used to study the BUP metabolism, whereas our study and the placental study conducted by Wang \textit{et al.}\textsuperscript{13} also utilised the mitochondrial fraction. According to the \( CL_{\text{int}} \) values, our results showed that the mitochondrial fraction is the most active liver subcellular fraction in BUP biotransformation, although the microsomal fraction was found to participate in BUP metabolism with a similar efficiency. Unfortunately, to the best our knowledge, no other study has investigated the \textit{in vitro} metabolism of BUP. The high participation of the mitochondrial fraction in BUP biotransformation in the liver is quite surprising, because mitochondria are not considered as major biotransformation organs. It has been demonstrated that some other drugs bearing a carbonyl group are also metabolized in the mitochondrial fraction (e.g. boceprevir, S-1360\textsuperscript{31,32}) but no specific participating enzyme has been identified yet. It is probably caused by poor knowledge of mitochondrial carbonyl-reducing enzymes. For example CBR4\textsuperscript{43} and likely DCXR\textsuperscript{34,35} belong to the group of mitochondrial carbonyl-reducing enzymes, but neither these nor any other relative mitochondrial enzymes are regarded as important biotransformation enzymes. Results concerning mitochondrial biotransformation could be highlighted for deeper study of mitochondrial carbonyl-reducing enzymes.

Because of the limited knowledge of mitochondrial enzymes, attention has been paid to ten important carbonyl-reducing enzymes that participate in the biotransformation of several xenobiotics in cytosolic and microsomal fractions.\textsuperscript{19,36,37} The screening performed to investigate their participation in BUP biotransformation \textit{in vitro} is demonstrated in \textbf{Figure 4}. Only five of the ten tested enzymes reduce BUP: cytosolic AKR1C1, AKR1C2, AKR1C3, and CBR1 and microsomal 11\( \beta \)-HSD1. The activity of microsomal 11\( \beta \)-HSD1 is approximately 3-fold and 80-fold higher compared with the activities of cytosolic AKR1C1 and CBR1, respectively, for the formation of TB (BUP concentration of 100 \( \mu \)M). These results are in accordance with the higher reductive activity for BUP found in the liver microsomal fraction compared with the liver cytosol. However, exact comparison of results of cytosolic enzymes in pure forms with 11\( \beta \)-HSD1 in the form of overexpressed S9 microsomes is little bit complicated, though a properly chosen control (microsomal fraction obtained after infection of S9 cells with wild-type virus) leads to adequate simulation of a single enzyme system that was demonstrated by a linear Eadie-Hofstee plot (\textbf{Fig. 5}). The participation of reductases in the metabolism of BUP has been suggested so far only on the basis of inhibition studies in the human liver and placental subcellular fractions.\textsuperscript{7,18} Our study provides the first demonstration of the utilisation of the recombinant forms of specific carbonyl-reducing enzymes and thus the direct identification of their participation in the BUP metabolism. The results of the inhibition studies on microsomes by 18\( \beta \)-glycyrhetinic acid\textsuperscript{7} and the incubation of recombinant 11\( \beta \)-HSD1 confirmed that 11\( \beta \)-HSD1 is a BUP reductase. The comparison of the \( K_M \) of the whole liver microsomal fraction with the \( K_M \) of the recombinant 11\( \beta \)-HSD1 revealed similar values; thus, 11\( \beta \)-HSD1 could be the main BUP reductase in human liver microsomes. However, this conclusion should be supported by other evidence, such as in the case of the identification of CYP2B6 as the main BUP hydroxylase.\textsuperscript{13} The identification of the cytosolic enzymes responsible for the formation of EB and TB is quite complicated. Through an indirect approach using inhibitors, a previous study\textsuperscript{71} identified CBRs as the enzymes that form EB and TB based on the strongest inhibitor of the cytosolic BUP reductase activity, menadione.\textsuperscript{80} In our study, we directly identified three additional cytosolic carbonyl reducing enzymes (AKR1C1, AKR1C2, and AKR1C3) as BUP reductases. The activity of CBR1, at least \textit{in vitro}, is very low in comparison to those of the other participating enzymes. But our results are probably not in conflict because menadione was described also as the substrate and thus potential competitive inhibitor of AKR1C enzymes.\textsuperscript{39,40} Moreover, the cellular concentrations of AKR1C1, AKR1C2, and AKR1C3 in the human liver are significantly lower compared with that of CBR1. Thus, even though these three enzymes display a significantly higher internal clearance, their contribution to the BUP metabolism \textit{in vivo} will be probably partial.\textsuperscript{28,41} The comparison of the \( K_M \) values of all of the BUP reductases with the \( K_M \) of CYP2B6 (\( K_M = 155.8 \pm 18 \mu \)M),\textsuperscript{13} which is the main BUP oxidase, revealed that CYP2B6 possesses higher affinity toward BUP than most carbonyl-reducing enzymes. This finding is one possible explanation for why the major metabolite \textit{in vivo} under normal conditions is HB and why the level of TB increases under pathological conditions.\textsuperscript{8,21}

In conclusion, the results of this study provide the first demonstration of the contributions and activities of specific human carbonyl-reducing enzymes in the biotransformation of BUP. Although the oxidative metabolite HB is regarded as the primary metabolite of BUP \textit{in vivo}, the reduced metabolite TB is the major metabolite produced by all of the hepatic subcellular fractions \textit{in vitro}. Surprisingly, the liver subcellular fraction that exhibits the most activity in the formation of BUP metabolites is the mitochondrial fraction, but the limited body of knowledge regarding mitochondrial carbonyl-reducing enzymes does not allow a detailed study. It was demonstrated that microsomal 11\( \beta \)-HSD1 and cytosolic AKR1C1 and CBR1 probably play significant roles in the formation of TB and EB in the human liver.

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\textbf{References}


