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Regular Article

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 BUP[8–10] and the elimination of TB and EB is slower in comparison with the parent drug.[2,9] 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

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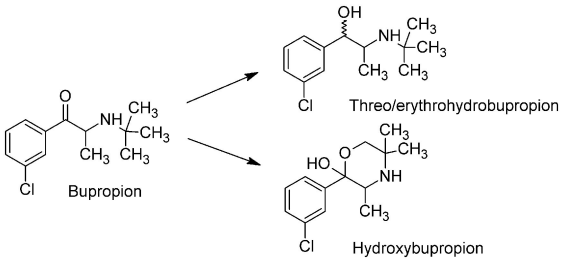

Fig. 1. Main biotransformation pathways of BUP: carbonyl reduction to erythrohydrobupropion and threohydrobupropion and hydroxylation to hydroxybupropion

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,[14,15] and other isoforms play a minor role in the formation of HB[16] or participate in the metabolism of BUP to form minor metabolites.[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 11β-hydroxysteroid dehydrogenases (11β-HSDs) and carbonyl reductases (CBRs) as enzymes that participate in the formation of TB and EB.[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.[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.[8] The TB concentration is sometimes even higher than that of HB (*e.g.*, in the cerebrospinal fluid[4] and in the plasma after overdose or abuse[21,22]), 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 $NADP^+$, glucose-6-phosphate dehydrogenase, magnesium chloride (Sigma-Aldrich), and glucose-6-phoshate 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 11β-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 (PAA, Pasching, Austria), and Sf9 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.[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.[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/XhoI 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 µg/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_{595} of 0.6 was achieved. Isopropyl β-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