INTERACTION OF BUPRENORPHINE AND ITS METABOLITE NORBUPRENORPHINE WITH CYTOCHROMES P450 IN VITRO

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ABSTRACT:

Buprenorphine (BUP) is a thebaine derivative used in the treatment of heroin and other opiate addictions. In this study, the selective probe reactions for each of the major hepatic cytochromes P450 (P450s) were used to evaluate the effect of buprenorphine and its main metabolite norbuprenorphine on the activity of these P450s. The index reactions used were CYP1A2 (phenacetin O-deethylation), CYP2A6 (cumarin 7-hydroxylation), CYP2C9 (diclofenac 4'-hydroxylation), CYP2C19 (omeprazole 5-hydroxylation), CYP2D6 (dextromethorphan O-demethylation), CYP2B6 (7-ethoxy-4-trifluoromethyl-coumarin 7-deethylation), CYP2E1 (chlorozoxzone 6-hydroxylation), and CYP3A4 (omeprazole sulfoxidation). Buprenorphine exhibited potent, competitive inhibition of CYP2D6 (Kᵢ, 10 ± 2 µM and 1.8 ± 0.2 µM) and CYP3A4 (Kᵢ, 40 ± 1.6 µM and 19 ± 1.2 µM) in microsomes from human liver and cDNA-expressing lymphoblasts, respectively. Compared with buprenorphine, norbuprenorphine demonstrated a lower inhibitory potency with CYP2D6 (22.4% inhibition at 20 µM norbuprenorphine) and CYP3A4 (13.6% inhibition at 20 µM) in microsomes from human cDNA-expressing lymphoblast cells. Furthermore, buprenorphine was shown to be a substrate of CYP2D6 (Kₘ = 600 µM; Vₘax = 0.40 nmol/min/mg protein) and CYP3A4 (Kₘ = 36 µM; Vₘax = 0.19 nmol/min/mg protein). The present in vitro study suggests that buprenorphine and its major metabolite norbuprenorphine are inhibitors of CYP2D6 and CYP3A4; however, at therapeutic concentrations they are not predicted to cause potentially clinically important drug interactions with other drugs metabolized by major hepatic P450s.

Buprenorphine (BUP) is a semisynthetic, highly lipophilic opioid derivative of the morphine alkaloid thebaine. As a partial opioid agonist, BUP has been used in the treatment of cocaine, heroin, and other opiate addictions (Kosten et al., 1989; Lewis et al., 1992; Mello et al., 1993; Vignau et al., 1998). Kosten et al. (1991) demonstrated a 33% reduction in illicit opioid use in opioid-dependent outpatients treated with BUP, with 72% treatment retention (Kosten et al., 1991). Preclinical studies in both primates and rodents have also indicated that BUP may reduce cocaine self-administration and attenuate place preferences (Kosten et al., 1991). The apparent reduced tendency to elicit withdrawal effects suggests that BUP may be an effective long-term agent in the treatment of cocaine, opioid, and perhaps other drug addictions. The positive utility of BUP, however, is tempered with its potential for abuse mainly in combination with benzodiazepines (Singh et al., 1992; Reynaud et al., 1998). The abuse of BUP is owing to its potent analgesic effects, which are 30 times that of morphine (Cowan et al., 1977). Fatalities have been reported in abusers of a high-dose, sublingual BUP formulation recently marketed in France for the substitution therapy of opiate addictions (Tracqui et al., 1997), as well as with concomitant BUP-benzodiazepine misuse (Reynaud et al., 1998).

In clinical practice, BUP is administered to drug-addicted patients concurrently receiving other drugs such as cocaine, morphine, benzodiazepines, and cyclizine (Singh et al., 1992; Teoh et al., 1993; Niv et al., 1998), several of which interact with cytochrome P450 (P450) enzymes. There is, therefore, a theoretical risk of drug interactions with respect to both inhibition of BUP metabolism and inhibition of metabolism of other drugs by BUP, which may lead to serious adverse consequences. The principal route of BUP metabolism in humans is via the N-dealkylation of its N-cyclopropylmethyl group to norbuprenorphine (Nor-BUP) (Iribarne et al., 1997). CYP3A4 has been shown to be the main enzyme responsible for BUP N-dealkylation in vitro in microsomes from human liver and cDNA-expressing cells (Iribarne et al., 1997; Kobayashi et al., 1998). At the time of this study, detailed inhibition profiles of either BUP or Nor-BUP with all of the major P450s had yet to be determined. During the course of preparation of this report, a study by Umehara et al. (2002) on the effect of BUP on the metabolism of P450 probe substrates was published, allowing comparisons to be made to the results of the present study.

Hepatic P450s 1A2, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1, and 3A4 are the most important P450 forms involved in drug metabolism in humans (Shimada et al., 1994). The metabolism of many therapeutically important drugs and endogenous compounds is mediated primarily by these enzymes. It is necessary to investigate the interactions of BUP and its main metabolite Nor-BUP with the major hepatic
P450s to fully evaluate potential clinically relevant drug-drug interactions. In addition to altering adverse event profiles, inhibition of drug metabolism by BUP may alter the efficiency of BUP as a treatment agent for addiction to these agents (e.g., benzodiazepines, codeine). In this study, the potency of inhibition of BUP and its main metabolite Nor-BUP toward the major hepatic P450s was evaluated in microsomes from human P450 cDNA-expressing lymphoblast cells and from human liver.

Materials and Methods

Chemicals and Reagents. Phenacetin, acetaminophen, coumarin, 7-hydroxycoumarin, 7-ethoxy-4-trifluoromethyl-coumarin, 7-hydroxy-4-trifluoromethylcoumarin, diclofenac sodium, dextromethorphan hydrobromide, dextrorphan, chlorzoxazone, budipine, pilocarpine, orphenadrine, sulfaphenazole, ketoconazole, α-naphthoflavone, diethylidithiocarbamate, and NADPH were purchased from Sigma-Aldrich (St. Louis, MO). BUP and Nor-BUP were kindly provided by the National Institute on Drug Abuse, Research Technology Branch (Rockville, MD). Omeprazole, 5-hydroxyomeprazole, and omeprazole sulfone were generously donated by Axel Härle, Möln达尔, Sweden. S(-)-N-Methenylthio, 6-hydroxycloazoxzone, and 4'-hydroxydiclofenac sodium were purchased from BD Gentest (Woburn, MA). All other chemicals and reagents used were of the highest commercially available quality.

Source of Microsomes. Microsomes from human lymphoblast cells expressing specific human P450s (1A2, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1, and 3A4) and pooled human liver microsomes were purchased from BD Gentest.

P450 Index Reaction Assays. Index reactions used were CYP1A2 (phenacetin O-deethylation), CYP2A6 (coumarin 7-hydroxylation), CYP2C9 (diclofenac 4'-hydroxylation), CYP2C19 (7-hydroxylation), CYP2D6 (dextromethorphan O-dealkylation), CYP2D6 (7-ethoxy-4-trifluoromethylcoumarin 7-deethylation), CYP2E1 (chlorozoxzone 6-hydroxylation), and CYP3A4 (omeprazole sulfonation). All incubations were carried out in 25 mM Tris-HCl buffer (pH 7.4) at 37°C in a shaking water bath. For each substrate, preliminary experiments were performed to determine whether metabolism formation was linear with respect to time, NADPH, and microsomal protein concentration. Incubation conditions were the same for both human liver microsomes and microsomes containing cDNA-expressed enzymes. Inhibitors were also incubated without substrate under the same conditions to ensure that the presence of inhibitor in the incubation would not interfere with the quantification of the respective metabolite. Incubation conditions (protein concentration, incubation time) and extraction/UV-high-performance liquid chromatography detection methods for all probe reactions were identical to those previously described (Zhang et al., 2001).

BUP Metabolism Assay. Briefly, the incubation mixture consisted of 100 μM Tris-HCl (25 mM, pH 7.4), 50 μM microsomes from cDNA-expressing cells or human liver (final protein concentration of 0.4 mg/ml), 50 μM BUP (5 μM, 10 μM, 25 μM, 50 μM, 100 μM, 250 μM, 500 μM, or 1000 μM), and 50 μM NADPH (final concentration of 1 mM). Incubations were carried out at 37°C for 30 min, with dextromethorphan (50 μM, 25 μM) as the internal standard. Samples were extracted with 1 ml of hexane/ether (4:1) and back-extracted into 10 mM HCl (200 μl) before high-performance liquid chromatography analysis (CSC-Spherisorb-phenyl column, 5 μm, 15 × 4.6 mm; isocratic mobile phase of acetonitrile/potassium phosphate buffer containing 1 mM octanesulfonic acid, 25:75, v/v, pH 3.8 at 1 ml/min; UV = 205 nm).

Chemical Inhibition Studies. Initial inhibition screening experiments of BUP and Nor-BUP with each P450 index reaction were carried out at two concentrations (20 μM and 200 μM) in microsomes from cDNA-expressing human lymphoblast cells and human liver. Substrate concentrations for each P450 probe reaction were identical to Kᵢ values determined in human liver microsomes (data not shown): 75 μM phenacetin (1A2) 1 μM coumarin (2A6), 6.25 μM 7-ethoxy-4-trifluoro-methyl-coumarin (2B6), 5 μM diclofenac sodium (2C9), 10 μM omeprazole (2C19), 5 μM dextromethorphan (2D6), 65 μM chlorzoxazone (2E1), and 30 μM omeprazole (3A4). Known inhibitors used as positive controls were: α-naphthoflavone (1A2), pilocarpine (2A6), orphenadrine (2B6), sulfaphenazole (2C9), S(+)-methenylthio (2C19), budipine (2D6), diethylidithiocarbamate (2E1), and ketoconazole (3A4), according to previously published reports (Rendic and Di Carlo, 1980; Bourrie et al., 1996; Eagling et al., 1998; Hichman et al., 1998). Of note, orphenadrine has not been shown to be CYP2B6-selective in previous studies (Gio et al., 1997; Sai et al., 2000); however, a more selective alternative CYP2B6 inhibitor is not readily available. BUP, Nor-BUP, and other inhibitors were prepared in methanol and evaporated prior to reconstitution in the incubate. In the case of orphenadrine, which has previously demonstrated mechanism-based P450 inactivation (Reidy et al., 1989), it was preincubated with microsomes and NADPH for 30 min before the addition of substrate.

For determination of apparent Kᵢ values in microsomes from cDNA-expression systems, final probe drug concentrations used for each index reaction were equal to 1/2 Kᵢ, Kᵢ, and 2 Kᵢ. At each of the substrate levels, metabolite formation was monitored in the absence and in the presence of BUP and Nor-BUP individually (at final inhibitor concentrations of 1/4 IC₅₀, 1/2 IC₅₀, IC₅₀, and 2 IC₅₀, with IC₅₀ referring to the concentration of inhibitor required to inhibit 50% of substrate metabolism at Kᵢ concentration). In those cases where the determined Kᵢ values were less than 25 μM, equivalent experiments with 30-min preincubation of BUP and Nor-BUP were conducted to determine whether there was evidence of mechanism-based inhibition. Kᵢ values were subsequently determined in pooled human liver microsomes using the same experimental design as in microsomes from cDNA-expression systems. All experiments were carried out in triplicate.

Data Analysis. Kᵢ and Vₘₐₓ values were determined by use of nonlinear regression analysis by Michaelis-Menten kinetics (rate of metabolism formation against substrate concentration) with Enzpack 3 software (Biosoft, Stapleford, Cambridge, UK). Inhibitory patterns were determined with Dixon plots. Kᵢ values for competitive inhibition were estimated through Dixon plots or by using Pharm/PCS software (Springer-Verlag, New York, NY). Means and standard deviations were calculated using Microsoft Excel (2000).

Results

Determination of Potency of Inhibition of Major P450s by BUP and Nor-BUP in Microsomes from cDNA-Expressing Lymphoblast Cells and Human Liver. Inhibition by BUP and Nor-BUP (at 20 and 200 μM) of the major hepatic P450s was evaluated in microsomes from cDNA-expressing cells (Fig. 1). BUP extensively inhibited CYP2D6 at both concentrations examined, with less than 10% of control activity remaining. CYP3A4 was also potently inhibited, to approximately 50% and 20% of control activity at 20 and 200 μM, respectively. P450 2A6, 2B6, 2C9, 2C19, and 2E1 were not significantly inhibited by BUP at 20 μM, with P450 2C19 and 2B6 both inhibited to approximately 40% of control at 200 μM BUP. P450 2A6, 2E1, and 2C19 were inhibited by only approximately 20% at 200 μM BUP. CYP1A2 was essentially not inhibited by BUP at either concentration studied.

As shown in Fig. 1B, Nor-BUP demonstrated the strongest inhibition of CYP2D6, with approximately 80% and 20% of control activity remaining at 20 and 200 μM, respectively. P450 3A4, 2C9, 2C19, and 2B6 were modestly inhibited by Nor-BUP at 200 μM. None of the other P450s studied were inhibited at either concentration investigated. In general, the degree of inhibition followed the same pattern in human liver microsomes as in microsomes from cDNA-expressing lymphoblast cells (Fig. 2), with BUP most potently inhibiting CYP2D6 of all P450s investigated.

In general, the potency of CYP2D6 inhibition by both BUP and Nor-BUP was attenuated in human liver (Fig. 2, A and B), in comparison with that observed in microsomes from cDNA-expressed P450s. Moreover, CYP2A6 was slightly more inhibited by Nor-BUP in human liver microsomes versus microsomes from cDNA-expressing lymphoblast cells, with approximately 60% remaining activity at 200 μM Nor-BUP.

Determination of the Potency of Inhibition of CYP2D6 by BUP and Nor-BUP. Because BUP and Nor-BUP demonstrated the greatest inhibition of CYP2D6 in initial screening experiments, CYP2D6 inhibition was further quantitated via determination of Kᵢ values.
As shown, BUP demonstrated potent inhibition of CYP2D6, with apparent $K_i$ values of 1.8 ± 0.2 μM and 10 ± 2 μM ($n = 3$) in microsomes from cDNA-expressing cells and human liver, respectively, confirming the inhibition screening data. The apparent $K_i$ for Nor-BUP of cDNA-expressed CYP2D6 was higher, as expected, at 42 ± 2.9 μM (Table 1).

To determine whether there was evidence of mechanism-based inhibition by BUP, the inhibition studies were also performed with a 30-min preincubation, in the absence of the probe substrate dextromethorphan (Table 1). The apparent $K_i$ values in cDNA-expressed CYP2D6 and pooled human liver microsomes after preincubation were 4.0 and 21 μM, respectively, similar to the $K_i$ values without preincubation (1.8 and 10 μM, respectively), indicating that mechanism-based inactivation by BUP is unlikely.

**Determination of the Potency of Inhibition of CYP3A4 by BUP.** BUP also inhibited CYP3A4 to a significant degree in screening experiments (Figs. 1A and 2A). To further evaluate this inhibition, apparent $K_i$ values of CYP3A4 with BUP were determined in microsomes from human liver and cDNA-expressing lymphoblast cells (Table 1). As indicated, BUP also demonstrated potent inhibition of CYP3A4, with apparent $K_i$ values of 19 ± 1.2 μM and 40 ± 1.6 μM in microsome from cDNA-expressing cells and human liver, respectively. After a 30-min preincubation in the absence of the probe substrate omeprazole, the apparent $K_i$ values in cDNA-expressed CYP2D6 and pooled human liver microsomes were 21 μM and 38 μM, respectively, similar to the results obtained without preincubation, again indicating a lack of mechanism-based inhibition by BUP.

**BUP Metabolism in Microsomes from CYP3A4- and CYP2D6-Expressing Lymphoblast Cells.** The demonstrated inhibition of CYP2D6 and CYP3A4 by BUP is indicative of an involvement of these enzymes in BUP metabolism. Therefore, the kinetics of BUP metabolism to Nor-BUP were evaluated in microsomes from CYP3A4- and CYP2D6-expressing lymphoblast cells (Table 2). As shown, BUP was metabolized to Nor-BUP by CYP3A4 with $K_m$ and $V_{max}$ values of 36 μM and 0.19 nmol/min/mg protein, respectively. This apparent $K_m$ value was similar to that obtained in human liver microsomes (33 μM). BUP was also metabolized to Nor-BUP by CYP2D6, with $K_m$ and $V_{max}$ values of 600 μM and 0.40 nmol/min/mg protein, respectively. These data suggest that BUP can bind to both CYP2D6 and CYP3A4 and predicts that BUP inhibition of both enzymes is likely competitive. Furthermore, Dixon plots (not shown) revealed competitive inhibition in both cases.

**Discussion**

In vitro kinetic and inhibition studies are useful tools for predicting in vivo pharmacokinetics and the potential for drug-drug interactions (Crespi et al., 1997). Through the use of such techniques with microsomes from both cDNA-expressing cells and human liver, we have...
demonstrated that BUP can effectively inhibit CYP2D6 and CYP3A4, and CYP3A4 is also capable of metabolizing BUP to its main metabolite, Nor-BUP. In addition, Nor-BUP potently inhibits CYP2D6. The importance of these two P450s in the metabolism of many clinically used drugs suggests that, in vivo, the likelihood of drug-drug interactions in those taking BUP should be examined.

Comparisons of the chemical structure of BUP with those of known specific CYP2D6 inhibitors including budipine, quinine, quindine, and lobeline, as well as other newer inhibitors previously identified in our laboratory, MPPP (1-methyl-4-phenyl-4-propionoxypiperidine) and PEPAP [1-(2-phenylethyl)-4-phenylacetoxy-piperidine] (Pritzker et al., 2002), show that they all contain the same pharmacophore “N-substituted piperidine” moiety. As molecular modeling studies of CYP2D6 have shown, the pharmacophore is mainly responsible for substrate-enzyme contact between the protonated nitrogen of the substrate and the carboxylic moiety of active site amino acid Asp301 in CYP2D6 (Ellis et al., 1995; Lewis et al., 1999). However, the high Km value (600 μM) and low intrinsic clearance (0.00067 ml/min/mg) observed currently with CYP2D6 suggest that although BUP can potently inhibit CYP2D6, it is not a good substrate of this enzyme. Such characteristics have been observed with other potent CYP2D6 inhibitors such as quinidine (Nielsen et al., 1999; Sai et al., 2000).

BUP was demonstrated to be a substrate of CYP3A4 in microsomes from cDNA-expressing lymphoblast cells (Km = 36 μM; Vmax/Km = 0.0054 ml/min/mg), with a similar apparent Km value (33 μM) observed in human liver microsomes. It would appear, therefore, that CYP3A4 is the major enzyme involved in BUP metabolism to Nor-BUP, which is in agreement with previous studies (Irizarbe et al., 1997; Kobayashi et al., 1998).

In general, as compared with BUP, Nor-BUP inhibited the P450s tested with a reduced potency. Studies with microsomes from CYP2D6-expressing lymphoblast cells clearly demonstrated that BUP was a more potent CYP2D6 inhibitor (Ki = 1.8 ± 0.2 μM) than Nor-BUP (Ki = 42 ± 2.9 μM). This reduced CYP2D6 inhibition may be due to effects of the secondary amine or tertiary amine on inhibitor-active site interactions; however, this is speculative. Detailed structure-function studies are necessary. Nor-BUP exhibited only 30% and 25% inhibition of CYP3A4 at a screening concentration of up to 200 μM in microsomes from cDNA-expressing cells and human liver, respectively, and inhibited CYP2C9 to a similar degree (Figs. 1 and 2). This was again lower than the CYP3A4 and CYP2C9 inhibition observed with BUP. The minor differences observed between cDNA-expressing and human liver microsomes may be in part due to inhibition of probe reactions mediated by more than one enzyme. For example, the N-deethylation of dextromethorphan in human liver microsomes is mediated by multiple enzymes, including CYP2D6 and 3A4 (Andersson et al., 1994; von Moltke et al., 1998); therefore, the selectivity of inhibition is likely reduced in human liver microsomes. As well, the sulfoxidation of omeprazole has been shown to be mediated by more than one enzyme (Andersson et al., 1994). Other factors, such as differences in the presence of enzyme cofactors, may also underlie the variation observed.

The present data are partially in agreement with the recently published data of Umehara et al. (2002). Both studies demonstrated that P450 2D6 and 3A4 are the primary enzymes inhibited by BUP in human liver microsomes. Our data, however, showed more potent inhibition of CYP2D6-dependent dextromethorphan metabolism (Ki = 10 μM) than CYP3A4-dependent omeprazole sulfonation (Ki = 40 μM), whereas the reverse was true with the results of Umehara et al. (2002) (CYP2D6 Ki = 21 μM; CYP3A4 Ki = 15 μM). The discrepancy in Ki values likely results from the different probe substrates utilized in the two studies (dextromethorphan versus bufuralol for CYP2D6; omeprazole versus testosterone for CYP3A4). The present study also investigated BUP interactions in microsomes from cDNA-expressing lymphoblast cells, where CYP2D6 was confirmed as the primary P450 inhibited by BUP. Moreover, our study has further characterized the inhibitory interactions of Nor-BUP and the kinetics of BUP metabolism, as discussed.

Characterizing the inhibition profiles of BUP and its active metabolite Nor-BUP with the major hepatic drug-metabolizing P450s provides the opportunity to predict actual drug interaction risk in vivo. The inhibitor concentrations used in these experiments are sufficiently high that if no inhibition is observed at 200 μM BUP or Nor-BUP, it is extremely unlikely that the enzyme will be inhibited in vivo. The present study indicated that BUP would be unlikely to demonstrate clinical interactions with drugs metabolized by P450s other than CYP3A4 or CYP2D6. Moreover, the relatively high Ki value observed with Nor-BUP toward CYP2D6 suggests that Nor-BUP would likely not inhibit any P450 to a significant degree at pharmacologically relevant concentrations. Concentrations of BUP in vivo in

### Table 1

Inhibition constants of buprenorphine and norbuprenorphine with CYP2D6 and CYP3A4 in microsomes from cDNA-expressing lymphoblasts and human liver

<table>
<thead>
<tr>
<th>P450</th>
<th>Inhibition Constant (Ki) (μM)</th>
<th>Buprenorphine without Preincubation (n = 3)</th>
<th>Buprenorphine with 30-min Preincubation (n = 1)</th>
<th>Norbuprenorphine without Preincubation (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human Liver Microsomes</td>
<td>Human Liver Microsomes</td>
<td>Human Liver Microsomes</td>
<td>Human Liver Microsomes</td>
<td></td>
</tr>
<tr>
<td>2D6</td>
<td>1.8 ± 0.2</td>
<td>10 ± 2</td>
<td>4.0</td>
<td>21</td>
</tr>
<tr>
<td>3A4</td>
<td>19 ± 1.2</td>
<td>40 ± 1.6</td>
<td>21</td>
<td>38</td>
</tr>
</tbody>
</table>

* Values are average ± S.D.

### Table 2

Kinetic constants of buprenorphine metabolism in vitro

<table>
<thead>
<tr>
<th>Enzyme System</th>
<th>Km (μM)</th>
<th>Vmax (nmol/min/mg)</th>
<th>Vmax/Km (ml/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human liver microsomes</td>
<td>33</td>
<td>1.6</td>
<td>0.049</td>
</tr>
<tr>
<td>CYP3A4-expressing microsomes</td>
<td>36</td>
<td>0.19</td>
<td>0.0054</td>
</tr>
<tr>
<td>CYP2D6-expressing microsomes</td>
<td>600</td>
<td>0.40</td>
<td>0.00067</td>
</tr>
</tbody>
</table>

* Microsomes from cDNA-expressing lymphoblast cells.
plasma after a single sublingual administration of an average 0.4-mg dose are typically between 0.45 ng/ml and 0.84 ng/ml (9.6 × 10^-7 - 1.7 × 10^-3 μM) (Tracqui et al., 1997). On the basis of the K_i values determined in this study, BUP (or Nor-BUP) at therapeutic concentrations of BUP may be altered by drugs metabolized by CYP3A4, leading likely to the predominant enzyme involved in BUP metabolism to its main degradation product. Crespi CL and Penman BW (1997) Use of cDNA-expressed human cytochrome P450 enzymes in multiple cytochrome P450 enzymes in human liver microsomes. J Biol Chem 272:20785–20805.


