Effect of Nonspecific Binding to Microsomes and Metabolic Elimination of Buprenorphine on the Inhibition of Cytochrome P4502D6

Shin UmEDa,*a,b Noriko HarAkaWa,a Masanori YaMaMoTo,a and Koichi UEnOb

*a Drug Discovery Research Department, Teijin Pharma Limited; 4–3–2 Asahigaoka, Hino, Tokyo 191–8512, Japan; and 
bDepartment of Geriatric Pharmacology and Therapeutics, Graduate School of Pharmaceutical Sciences, Chiba University; 1–8–1 Inohana, Chiyuo, Chiba 260–8675, Japan.

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Recently, the pharmaceutical industry has employed the high-throughput method for the evaluation of cytochrome P450 (CYP) inhibition, using a combination of the heterologously expressed enzyme and a fluorogenic substrate. When buprenorphine (BN), a potent mixed agonist-antagonist analgesic, was evaluated by this method, it exhibited potent inhibition of CYP2D6 with an IC50 value of 0.25 μM in recombinant CYP2D6-expressing insect cell microsomes (rCYP2D6 microsomes). In contrast, the IC50 value was 22.7 μM in human liver microsomes (HLM) using a classical method. Although the substrate concentrations in each study were set to near the Km values, there was a large discrepancy in IC50 values. When we investigated the effect of nonspecific binding to microsomes on the inhibitory potency, with a view to clarifying this discrepancy, the unbound fraction in microsomes (fu,mic) was 0.06—0.21 and 0.99 in HLM and rCYP2D6 microsomes, respectively. The corrected IC50 value (1.74 μM) using free BN concentrations was much smaller than the uncorrected value. On the other hand, it was observed that the concentration of BN in HLM decreased rapidly due to metabolism by CYP3A4 while that in rCYP2D6 microsomes decreased only slightly. We then investigated the effect of incubation time on the inhibitory potency, since the rapid elimination of BN in HLM could have been a cause of the discrepancy. The IC50 value for BN was observed to decrease slightly from 22.7 to 17.1 μM, following the shortening of the incubation time from 10 to 2 min in HLM. We conclude that nonspecific binding to microsomes of the inhibitor could affect the inhibitory potency against CYP2D6. If this factor is considered, a more precise estimate of the risk of adverse drug interaction could be achieved.

Key words  buprenorphine; CYP2D6 inhibition; nonspecific binding to microsome

The inhibition of drug-metabolizing enzymes, such as cytochrome P450 (CYP) often can lead to alterations in the extent of exposure to coadministered drug. Such drug interactions can limit the use of a drug because of adverse clinical effects, depending upon the potential for the toxicity of affected drug. Since CYP2D6 is one of the important human CYP isozymes and metabolizes more than 20% of commercially available drugs, a profound understanding of the inhibitory potency against CYP2D6 of the drug candidates is important in the discovery and development of new drugs. The pharmaceutical industry routinely has assessed the risk of adverse drug interaction by determining the inhibitory effect of the candidates on the rate of a probe reaction that represents of a specific CYP enzyme activity in human liver microsomes (HLM). The in vitro findings obtained with one probe substrate are usually extrapolated to the potential of compound to affect all substrates of the same enzyme. Bufuralol (BF) 1'-hydroxylation and dextromethorphan (DM) O-demethylation have generally been used as probe reactions for the evaluation of CYP2D6 inhibition.

Recently many pharmaceutical companies have used high throughput methods for the evaluation of CYP inhibition using a combination of the recombinant CYP and the non-fluorescent probe substrate that produces fluorescent throughput methods for the evaluation of CYP inhibition. O-demethylation have generally been used as probe reactions for the evaluation of CYP2D6 inhibition.

When we evaluated the inhibition by BN against CYP2D6 using the high throughput method in the present study, BN strongly inhibited it (IC50=0.25 μM). Although the substrate concentration (1.5 μM) in this assay was near to the reported Km value (1.0 μM) for this reaction, this IC50 value showed a large discrepancy with the above reported Km values in HLM.

Margolis et al. reported that the inhibitory potency of some highly lipophilic drugs against CYP2D6 decreased with an increase in microsomal concentration. This is caused by the decrease of free concentrations of inhibitors due to an increase in the nonspecific binding to microsomes. As BN shows high lipophilicity, it could have high binding affinity to microsomes. Thus, we considered nonspecific binding to be a probable cause of the discrepancy.

On the other hand, BN is rapidly metabolized by CYP3A4, but not by CYP2D6 at low substrate concentrations.
Consequently, in inhibition studies with BN, its concentration could decrease in HLM containing CYP3A4, but not in rCYP2D6 microsomes. We also considered elimination rate of the inhibitor to be a probable cause of the discrepancy in inhibitory potency between the two systems.

Therefore, to clarify this discrepancy we investigated the effect of nonspecific binding to microsomes and the metabolic elimination of BN, on the inhibition of CYP2D6.

MATERIALS AND METHODS

Chemicals  Buprenorphine·HCl and norbuprenorphine (NBN) were supplied by Teysan Pharmaceuticals (Tokyo, Japan). 3-[2-(N,N-Diethyl-N-methylammonium)methyl]-7-methoxy-4-methylcoumarin (AMMC) and 3-[2-(diethylamino)ethyl]-7-hydroxy-4-methylcoumarin hydrochloride (AHMC) were purchased from BD Gentest (Woburn, MA, U.S.A.). Dextromethorphan (DM) was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Dextrophan (DP) was purchased from Ultrafine Chemicals (Manchester, U.K.). All other chemicals were of high purity and were commercially obtained.

Source of Microsomes  Recombinant CYP2D6-expressing insect cell microsomes (rCYP2D6 microsomes) were obtained from BD Gentest. These were coexpressed with NADPH-CYP reductase. Pooled human liver microsomes (HLM) from 30 donors (19 male and 11 female donors) were obtained from Xeno Tech LLC (Kansas City, KS, U.S.A.). This pool was prepared from different amounts of liver microsomes from each donor to standardize each enzyme activity for major CYP isoforms (including CYP2D6 and CYP3A4) representative of an overall population at the supplier.

Inhibition Study in rCYP2D6 Microsomes  The IC\textsubscript{50} values for BN and NBN in rCYP2D6 microsomes were determined by measuring AMMC O-demethylation activity, quantified as its fluorescent metabolite, AHMC. The reaction mixture contained 7.5 pmol/ml rCYP2D6 microsomes (0.05 mg protein/ml), 100 mM potassium phosphate buffer (pH 7.4), 0.41 mM MgCl\textsubscript{2}, 0.41 mM G-6-P, 0.4 units/ml G-6-PDH, 8.2 \mu M NADPH, 1.5 \mu M AMMC, and various concentrations of BN (0.08—100 \mu M) or NBN (0.8—100 \mu M) in a final volume of 0.2 ml in each well of a 96-well plate. The plates were incubated at 37 °C for 45 min. The reaction was terminated by the addition of 1.5 ml acetonitrile. After the termination of incubation, mixtures were centrifuged at 3000 rpm for 10 min and the supernatant was evaporated to dryness. All samples were dissolved in the mobile phase of a HPLC system which was used for analysis. Incubations for 2 min were also carried out to evaluate the effect of incubation time.

Determination of Nonspecific Binding of BN to Microsomes  Nonspecific binding of BN to HLM and rCYP2D6 microsomes was determined by equilibrium dialysis. The incubation mixtures without substrates and NADPH (NADP\textsuperscript{+} in rCYP2D6 microsomes) used for the inhibition study described above, were also used in the dialysis. The concentrations of BN in HLM and rCYP2D6 microsomes were 1—125 \mu M and 1 \mu M, respectively. Dialysis was conducted using an Equilibrium Dialyzer (Spectrum Industries, Los Angeles, CA). Spectra/Por no.2 membranes, with molecular mass cutoff of 12—14 kDa were used and the cells were rotated at 20 rpm for 6 h at 37 °C. All samples were analyzed by HPLC.

Metabolic Elimination of BN in HLM and rCYP2D6 Microsomes  The metabolic elimination of BN in HLM and rCYP2D6 microsomes was determined by measuring the concentrations of BN in the inhibition study mixture described above (excluding the substrate) by HPLC-UV. The initial concentration of BN in each mixture was 5 \mu M. The time-course for each BN concentration (1—125 \mu M) in the inhibition study in HLM was also measured by HPLC.

HPLC Analysis  The HLM incubations were analyzed for the metabolites of DM by the HPLC-fluorescent method. The HPLC system consisted of a LC-10A system (Shimadzu, Kyoto) and a 4.6×150 mm ODS-80Ts column (Tosoh, Tokyo). The mobile phase in the inhibition study was 0.025% TFA in a mixture of acetonitrile/H\textsubscript{2}O (28:72). The flow rate for analysis was 1.5 ml/min. Fluorescent detection of DP was performed at excitation and emission wavelengths of 270 nm and 312 nm, respectively. The column temperature was maintained at 37 °C. The mobile phase for the measurement of BN in the metabolic elimination and microsomal binding studies was 0.05% TFA in a mixture of acetonitrile/H\textsubscript{2}O (1:2) at a flow rate of 1 ml/min. UV absorption was monitored at 220 nm. The column temperature was 40 °C.

Data Analysis  The apparent K\textsubscript{m} value for DM O-demethylation was determined from the formation rate of substrate metabolite over the above mentioned substrate concentration ranges and were estimated by linear regression analysis of Eadie–Hofstee plots. The IC\textsubscript{50} values were determined by semi-log plotting over two orders of magnitude of the concentrations of BN (0.08—125 \mu M) or NBN (0.4—125 \mu M) on the x-axis versus percentage of remaining activity on the y-axis. The fu\textsubscript{mic} value was calculated by the following equation: fu\textsubscript{mic} = Cb/(Cm-Cb)(V1/V0)+Cb, where Cb, Cm are the concentrations of compounds in buffer and microsomal sample cell after dialysis, respectively, V0 is the initial volume of the buffer cell sample, and V1 is the volume of the microsomal cell sample after dialysis. The microsomal protein binding ratio was calculated by the following equation: binding ratio (%) = 100×(1−fu\textsubscript{mic}).
RESULTS

Inhibition by BN and NBN in HLM and rCYP2D6 Microsomes  
The reported values for apparent $K_m$, substrate concentration of probe reactions and inhibitory potencies ($IC_{50}$ and $K_i$) of BN against CYP2D6 in HLM and rCYP2D6 microsomes are shown in Table 1. The apparent $K_m$ value for DM O-demethylation was similar to the previously reported value. In rCYP2D6 microsomes, BN exhibited strong inhibition of AMMC O-dealkylation with an $IC_{50}$ value of 0.25 $\mu M$. (Table 1, Fig. 1). In HLM, BN exhibited relatively strong inhibition of CYP2D6-mediated DM O-demethylation with an $IC_{50}$ value of 22.7 $\mu M$ (Table 1, Fig. 2). Calculated $K_i$ values for inhibition of DM O-demethylation by BN in HLM were similar to those reported experimentally. In contrast, this value for AMMC O-dealkylation was much smaller than other values (Table 1). NBN also exhibited a weak inhibition of CYP2D6, with $IC_{50}$ values of 68.9 $\mu M$ and >125 $\mu M$ in rCYP2D6 microsomes and HLM respectively; however these inhibitory potencies were lower than those of BN (Figs. 1, 2).

Effect of Nonspecific Binding of BN to Microsomes on Inhibitory Potency  
BN bound strongly to HLM with $fu_{mic}$ values of 0.06—0.21 at 1—125 $\mu M$, but bound to rCYP2D6 to an extremely small extent ($fu_{mic}$ value of 0.99 at 1 $\mu M$) (Table 2). The $IC_{50}$ value corrected using free concentrations based on their $fu_{mic}$ values in HLM was 1.74 $\mu M$, which is about 13-fold smaller than the value of 22.7 $\mu M$ obtained from total concentrations (Fig. 3).

Metabolic Elimination of BN in HLM and rCYP2D6 Microsomes  
The metabolic elimination of BN in HLM and rCYP2D6 microsomes is shown in Fig. 4. BN (5 $\mu M$) was rapidly eliminated in HLM within 10 min, the incubation time used in the inhibition study. However, its elimination in rCYP2D6 microsomes was negligible, even after 45 min.

Effect of Incubation Time on Inhibitory Potency  
$IC_{50}$ values for two different incubation times in HLM were measured to investigate the effect of possible decreases in inhibitor concentration due to metabolism of BN. The $IC_{50}$ value slightly decreased from 22.7 to 17.1 $\mu M$, following shortening of the incubation time from 10 to 2 min (Fig. 5). When the concentrations of BN in the inhibition

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>Probe reaction</th>
<th>$K_m$ ($\mu M$)</th>
<th>$[s]^{a}$ ($\mu M$)</th>
<th>$IC_{50}$ ($\mu M$)</th>
<th>$K_i$ ($\mu M$)</th>
<th>Ref.</th>
</tr>
</thead>
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<tr>
<td>rCYP2D6</td>
<td>AMMC O-dealkylation</td>
<td>1.0$^{b}$</td>
<td>1.5</td>
<td>0.25</td>
<td>0.1$^{c}$</td>
<td>—</td>
</tr>
<tr>
<td>HLM</td>
<td>Dextrorhemorphan O-demethylation</td>
<td>5.4</td>
<td>4.0</td>
<td>22.7</td>
<td>13$^{c}$</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Bufuralol 1’-hydroxylation</td>
<td></td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>Dextrorhemorphan O-demethylation</td>
<td>5.0</td>
<td>—</td>
<td>10</td>
<td>12</td>
<td></td>
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</tbody>
</table>

Each experiment was performed in duplicate. $a$) $[s]$ are substrate concentrations in inhibition studies for determination of $IC_{50}$ values. $b$) Apparent $K_m$ value of AMMC O-dealkylation is quoted from ref. (4). $c$) $K_i$ values were calculated by assuming competitive inhibition using the following equation: $K_i=(K_m \times IC_{50})/(K_m+[s])$.

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>BN ($\mu M$)</th>
<th>Binding ratio (%)</th>
<th>$fu_{mic}$</th>
<th>BN free ($\mu M$)</th>
</tr>
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<tr>
<td>HLM</td>
<td>1</td>
<td>79.0</td>
<td>0.21</td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>88.2</td>
<td>0.12</td>
<td>0.59</td>
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<tr>
<td></td>
<td>25</td>
<td>92.4</td>
<td>0.08</td>
<td>1.89</td>
</tr>
<tr>
<td></td>
<td>125</td>
<td>94.4</td>
<td>0.06</td>
<td>7.01</td>
</tr>
<tr>
<td>rCYP2D6</td>
<td>1</td>
<td>0.66</td>
<td>0.99</td>
<td>0.99</td>
</tr>
</tbody>
</table>

Each experiment was performed in duplicate. Each difference between duplicates was lower than 5% of the mean value.

Fig. 1. The Inhibitory Effect of BN and NBN in rCYP2D6 Microsomes  
Inhibition of rCYP2D6 by BN (■) and NBN (▲) was determined as AMMC O-dealkylation. Each experiment was performed in duplicate and each value is shown.

Fig. 2. The Inhibitory Effect of BN and NBN against CYP2D6 in HLM  
Inhibition by BN (■) and NBN (▲) in HLM was determined as dextrorhemorphan O-demethylation. Each experiment was performed in duplicate and each value is shown.

Fig. 3. The Effect of Non-specific Binding to Microsomes on BN Inhibition in HLM  
Inhibitory potencies ($IC_{50}$ values) of BN against CYP2D6 in HLM were calculated based on total (■) and free (▲) concentrations of BN. The concentrations corresponding to the arrows denote $IC_{50}$ values. Each experiment was performed in duplicate.
we investigated the effect of nonspecific binding to microsomes and the metabolic elimination of BN on CYP2D6 inhibition, to clarify this discrepancy. They explained the difference in terms of metabolism by other CYPs in HLM. Donato et al. also reported a similar case. They explained the difference in terms of metabolism by flavin-containing monooxygenase that exists only in HLM. Thus, the possibility that the difference in elimination rate of the inhibitor in HLM and rCYP2D6 microsomes affects inhibitory potency cannot be excluded. In such cases the inhibition study in HLM is also necessary.

In the present study, we focused on nonspecific inhibitor binding. Venkatakrishan et al. have reported that nonspecific binding of substrate also affects the apparent $K_{m}$ values for its metabolism. Foti et al. reported that bufuralol, a typical probe substrate of CYP2D6, binds to HLM. As substrate kinetics contribute to the inhibition constant ($K_{i}$), nonspecific binding of substrate to microsomes should also be considered for more precise evaluation of the inhibition.

BN was rapidly metabolized to NBN by CYP3A4 in HLM but not in rCYP2D6 microsomes (Fig. 4) and NBN showed much weaker inhibition of CYP2D6 (Figs. 1, 2). Therefore we considered that this rapid elimination by CYP3A4 in only HLM was the cause of the discrepancy. Since the residual concentration of BN in HLM rapidly decreased through the incubation time in the metabolic elimination study (Fig. 4), we investigated the effect of shortening the incubation time on inhibitory potency in HLM. However, the IC$_{50}$ value in HLM only slightly decreased from 22.7 to 17.1 µM with shortening of the incubation time (Fig. 5). Indeed, an initial BN concentration of 25 µM (near to the IC$_{50}$ value) decreased to 76% and 31% of the initial concentration at 2 and 10 min respectively (Fig. 6) and the change in residual inhibitor concentration over these incubation times was not significant at this concentration of BN. The reported apparent $K_{m}$ value of BN $N$-dealkylation in HLM is about 39 µM. Hence it was considered that the elimination rate of BN decreased in a manner depending on its concentration due to the saturation of this reaction.

In the present study, the effect of the metabolic elimination of BN was not so clear. However, in a similar comparison, Palamanda et al. found that some compounds exhibited lower IC$_{50}$ values against rCYP2D6 microsomes than in HLM. They speculated that these differences occurred because of a decrease in inhibitor concentration due to metabolism by other CYPs in HLM. Donato et al. reported a similar case. They explained the difference in terms of metabolism by flavin-containing monooxygenase that exists only in HLM. Thus, the possibility that the difference in elimination rate of the inhibitor in HLM and rCYP2D6 microsomes affects inhibitory potency cannot be excluded. In such cases the inhibition study in HLM is also necessary.

We therefore suggest that the results of inhibition studies in both systems should be compared for more appropriate evaluation of CYP2D6 inhibition. Especially in HLM, nonspecific binding of inhibitor and substrate to microsomes should be considered.
Uehara et al. and Zhang et al. have estimated the possibility of clinical drug interaction of BN on the bases of the $K_i$ values in their studies.\textsuperscript{11,12} However, it was considered that their $K_i$ values might be over estimated, from the result of the present study. Accordingly, we also discussed this possibility. BN is extensively metabolized by the first-pass, and its oral bioavailability is estimated to be 15%.\textsuperscript{13} Therefore, to avoid this first-pass metabolism, BN is clinically administered by multiple routes (e.g. injection, sublingual, suppository) excluding the oral route. BN at relatively higher doses has been proposed for the management of opioid addicts\textsuperscript{19,20}.

According to Sawada, the degree of maximum increase ($R$) in $AUC$ of coadministered drugs caused by the drug interaction, when elimination clearance consists of only one route by hepatic clearance, can be calculated using the following equation: $R=1+(C_{LI}/K_i)$.\textsuperscript{21} In this equation, $C_{LI}$ denotes the unbound concentrations of the inhibitor in the region of the metabolizing enzyme. As BN is administered by non-oral routes, its plasma concentration in the portal vein does not differ to that in the systemic circulation. The maximum plasma concentration ($C_{max}$) after administration of 4 mg of a sublingual formulation of BN is 3.29±1.01 ng/ml (mean±S.E.).\textsuperscript{22} Accordingly, the $C_{max}$ even in the maximum dosage of this formulation (16 mg) was estimated to be approximately 15 ng/ml. Binding of BN to plasma protein in humans is 94.4—96.7%.\textsuperscript{23} Consequently, the free plasma concentration of BN was approximately 0.5 ng/ml (0.001 μM). This value was more than 100-fold lower than the smallest calculated $K_i$ value (0.1 μM: in rCYP2D6 microsomes) of our results. Therefore, if the free plasma BN was not concentrated by more than 100-fold in the hepatocytes, this would not change the plasma levels of coadministered drugs due to inhibition of CYP2D6-mediated hepatic metabolism. Indeed, even in the case of high dosage, no adverse drug interaction concerning CYP2D6 has been reported. It was considered unlikely therefore, that BN would cause a drug interaction, because of its high plasma protein binding and non-oral administration route.

In conclusion, we have shown that nonspecific binding of BN to microsomes could affect the inhibitory potency towards CYP2D6. Taking such factors into consideration, a more precise estimation of the risk of adverse drug interaction can be achieved.

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