Short Communication

VALIDATION OF (−)-N-3-BENZYL-PHENOBARBITAL AS A SELECTIVE INHIBITOR OF CYP2C19 IN HUMAN LIVER MICROSONES

Received October 30, 2003; accepted February 26, 2004

This article is available online at http://dmd.aspetjournals.org

ABSTRACT:

(−)-N-3-Benzyl-phenobarbital (NBPB) was reported to be a potent and selective inhibitor of CYP2C19. To validate the selectivity of NBPB toward CYP2C19 in human liver microsomes, the inhibitory effects on major cytochrome P450 isoform-specific reactions were evaluated in the present study. In human liver microsomes, NBPB showed potent competitive inhibition on CYP2C19-mediated S-mephenytoin 4'-hydroxylation with an IC_{50} value of 0.25 μM and K_i value of 0.12 μM, whereas weak inhibition was observed for CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2D6, and CYP3A4-mediated reactions with IC_{50} values >100, >100, 62, 34, 19, >100, and 89 μM, respectively. Importantly, its selectivity toward CYP2C19 among the CYP2C subfamily was demonstrated. Therefore, NBPB can be used as a potent and selective inhibitor to establish the relative contribution of CYP2C19 for in vitro reaction phenotyping studies. This compound can also serve as a positive control inhibitor of CYP2C19 for routine screening of P450 reversible inhibition when human liver microsomes are used as the enzyme source.

Cytochrome P450 2C19 (CYP2C19) is known to be a genetically polymorphic enzyme responsible for the 4’-hydroxylation of S-mephenytoin in humans (Wrighton et al., 1993a; Goldstein et al., 1994). “Poor metabolizers” with low CYP2C19 activity represent 3 to 5% of Caucasians and African-Americans, and 12 to 23% of most Asian populations (Goldstein, 2001). Genetic polymorphism in CYP2C19 has been shown to affect the clearance of many clinically important drugs, such as diazepam, imipramine, propranolol, S-mephenytoin, omeprazole, lanzoprazole, and pantoprazole (Wedlund, 2000; Goldstein, 2001). As a result, it is essential to identify the possible involvement of CYP2C19 in the metabolism of new therapeutic agents in the early stages of drug development. In vitro cytochrome P450 reaction phenotyping can successfully predict potential drug interactions and the polymorphic impact on drug metabolism and disposition (Lin and Lu, 1998; Dahl, 2002; Rodrigues and Rushmore, 2002). Inhibition of the in vitro metabolism of a drug in human liver microsomes by isoform-selective inhibitory antibodies and chemical inhibitors is a valuable approach for reaction phenotyping to identify which human P450 enzymes are responsible for the metabolism of a drug (Wrighton et al., 1993b; Rodrigues, 1999; Lu et al., 2003). Many commonly used chemical inhibitors for CYP2C19, such as trimethylammoniumpropylmethysulfate, omeprazole, nootkatone, and ticlopidine are not completely selective because they also inhibit one or more other P450 isoforms (Ko et al., 1997, 2000; Tassaneeyakul et al., 2000; Zhang et al., 2001). Recently, (+)-N-3-benzyl-nirvanol and NBPB were synthesized and found to be highly potent competitive inhibitors of CYP2C19-mediated 3-O-methylfluorescein demethylation in the presence of recombinant CYP2C19 (Suzuki et al., 2002). In addition, the inhibitory effects of (+)-N-3-benzyl-nirvanol and NBPB on CYP2C19-mediated S-mephenytoin 4’-hydroxylation were determined in human liver microsomes, with K_i values ranging from 210 to 280 nM and 71 to 94 nM, respectively. Although the effects of 1 μM (+)-N-3-benzyl-nirvanol and 0.3 μM NBPB on the activities of a panel of cDNA-expressed P450 isoforms were examined, the selectivities of these two compounds were not evaluated in human liver microsomes. Walsky and Obach (2003) recently published a Letter to the Editor of Drug Metabolism and Disposition confirming the potency and selectivity of (+)-N-3-benzyl-nirvanol as a CYP2C19 inhibitor in human liver microsomes. In the present study, NBPB was synthesized and its selectivity was evaluated in human liver microsomes.

Materials and Methods

Materials. Phenacetin, diclofenac, 4’-hydroxybutyranilide, flufenamic acid, cortisone, testosterone, 6β-hydroxytestosterone, 7,8-benzoflavone, paclitaxel, baccatin III, quercetin, coumarin, 7-hydroxy coumarin, tranylcypromine, N-(α-methylbenzyl)-1-aminobenzotriazole, phenobarbital sodium, glucose 6-phosphate, NADP^+, and glucose-6-phosphate dehydrogenase were purchased from Sigma-Aldrich (St. Louis, MO). Acetaminophen, quinidine, propranolol, phenobarbital, benzyl bromide, and anhydrous N,N-dimethyl formamide were obtained from Aldrich Chemical Co. (Milwaukee, WI). Bufuralol, 1’-hydroxy-bufuralol, 4’-hydroxydiclofenac, S-mephenytoin, 4’-hydroxymephenytoin, bupropion, hydroxybupropion, and 6α-hydroxypaclitaxel were purchased from BD Gentest (Woburn, MA). Ketoconazole was obtained from Sigma/RBI (Natick, MA). Sulfaphenazole was synthesized at Merck Research Laboratories. Pooled human liver microsomes (from 10 male and 10 female donors) were purchased from Tissue Transformation Technologies (Edison, NJ).

Synthesis of NBPB. NBPB was synthesized according the procedure described by Suzuki et al. (2002). Briefly, phenobarbital sodium was reacted with benzyl bromide in anhydrous N,N-dimethyl formamide to generate (±)-N-3-benzyl-phenobarbital. The pure enantiomers were isolated by chiral HPLC. The chemical structure and chemical purity (>99%) were established by
LC-MS/MS and NMR. The chirality of NBPB was determined to be 99% by chiral HPLC as described in the above publication.

Microsomal Incubations. An aliquot from a pool of liver microsomes was incubated at 37°C in a reaction mixture (final volume, 0.2 ml) containing the appropriate P450 probe substrate and NBPB (0.05–100 µM). In each case, the reaction mixture contained 100 mM potassium phosphate buffer (pH 7.4), 3 mM MgCl₂, and an NADPH-regenerating system. The substrate concentrations (near to Kᵣ values), microsomal protein concentrations, and incubation times were as follows: phenacetin O-deethylation (100 µM, 0.5 mg/ml, 20 min), coumarin 7-hydroxylation (1 µM, 0.1 mg/ml, 8 min), bupropion hydroxylation (100 µM, 0.5 mg/ml, 20 min), paclitaxel 6α-hydroxylation (15 µM, 0.5 mg/ml, 20 min), diclofenac 4'–hydroxylation (10 µM, 0.25 mg/ml, 10 min), S-mephenytoin 4’-hydroxylation (80 µM, 0.5 mg/ml, 20 min), bufuralol 1’-hydroxylation (15 µM, 0.25 mg/ml, 10 min), and testosterone 6β-hydroxylation (50 µM, 0.25 mg/ml, 10 min). For time-dependent inhibition studies, human liver microsomes were preincubated with 5, 10, and 20 µM NBPB at different times ranging from 5 to 30 min at 37°C. The incubations were diluted 10-fold with 0.1 M potassium phosphate buffer (pH 7.4) containing 400 µM S-mephenytoin and an NADPH-generating system. The incubations were allowed to continue for an additional 20 min.

LC-MS/MS Analyses. All incubations except coumarin 7-hydroxylation were terminated by adding 0.4 ml of 75:25 acetonitrile/water (v/v) containing 0.05% formic acid. This solution also contained the internal standard for each analyte. chromatographic separation was achieved using a Zorbax SB C8 column (4.6 mm i.d.) with a mobile phase consisting of a mixture of buffer A (10 mM ammonium acetate, pH 4.0) and buffer B, 0.05% formic acid in acetonitrile. The analytes were eluted using a linear gradient from 5% to 90% B over 4.2 min at a flow rate of 1.5 ml/min at ambient temperature.

HPLC Analysis. Incubations for coumarin 7-hydroxylation were terminated by adding 0.2 ml of methanol. The samples were centrifuged at 14,000g for 10 min, and aliquots (50 µl) of the supernatants were analyzed by Agilent HPLC for 40 min with a gradient of 95:5 to 50:50 methanol:water, and B, 0.05% formic acid in acetonitrile. The resulting samples were centrifuged at 3000g for 10 min at 4°C, and 0.2-ml aliquots of the supernatants were removed to a new tube that contained 0.1 ml of 0.05% formic acid in water. These samples then were subjected to LC-MS/MS analysis on an API 3000 triple quadrupole mass spectrometer (PerkinElmerSciex Instruments, Boston, MA). The instrument was operated in positive ionization mode using the Heated Nebulizer interface. Selected reaction monitoring was used to determine specific precursor-ion to product-ion transitions for each analyte. Chromatography was conducted using an Aquasep reversed-phase HPLC column (2 mm i.d. × 50 mm, 5 µm; ES Industries, West Berlin, NJ). The mobile phase consisted of A, 0.05% formic acid in deionized water, and B, 0.05% formic acid in acetonitrile. The analytes were eluted using a linear gradient from 5 to 90% B over 4.2 min at a flow rate of 1.5 ml/min at ambient temperature.

Data Analysis. Each assay was performed in triplicate. The IC₅₀ values, calculated by nonlinear regression analysis using KaleidaGraph software (KaleidaGraph Synergy Software, Reading, PA), were used to express the relative inhibitory potency of NBPB. The Ki value was calculated by nonlinear regression analysis using Sigmaplot Enzyme Kinetics Module 1.1 (SPSS Inc., Chicago IL).

Results

To assess the selectivity of NBPB on CYP2C19 among the CYP2C subfamily enzymes, the inhibitory effects of NBPB on CYP2C19-mediated S-mephenytoin 4’-hydroxylation, CYP2C9-mediated diclofenac 4’-hydroxylation, and CYP2C8-mediated paclitaxel 6α-hydroxylation were determined in human liver microsomes. The selectivity can be seen in Fig. 1, where a concentration of NBPB equal to its apparent IC₅₀ value for CYP2C19 (0.25 µM) produced virtually no inhibitory effect for CYP2C9 (diclofenac 4’-hydroxylation) and CYP2C8 (paclitaxel 6α-hydroxylation). When evaluated at 6.25 µM (25-fold the IC₅₀ value for CYP2C19), the percentage of inhibition of the activity of CYP2C19, CYP2C9, and CYP2C8 was 93, 25, and 16%, respectively. The inhibitory potencies for these reactions were further determined using a range of NBPB concentrations (0.05–100 µM). The resulting IC₅₀ values, as listed in Table 1, were 0.25, 19, and 34 µM for the reactions catalyzed by CYP2C19, CYP2C9, and CYP2C8, respectively. Since the IC₅₀ values for CYP2C9 and CYP2C8 were about 70- to 140-fold higher than for CYP2C19, the results again demonstrate that NBPB has high selectivity for CYP2C19 among closely related CYP2C subfamily members in human liver microsomes.

Suzuki et al. (2002) reported that NBPB inhibited CYP2C19-mediated S-mephenytoin 4’-hydroxylation in a competitive manner. To verify the type of inhibition, a range of substrate concentrations (5–100 µM) and various concentrations of NBPB (0–0.6 µM) were used to determine the kinetic parameters for the reactions. An increase in Kᵣ values and no change in Vₘₐₓ values in the presence of NBPB confirmed that the inhibition pattern is a typical competitive type. The Ki value was determined to be 0.12 µM using a simple competitive inhibition equation. In addition, to investigate whether NBPB would cause a time-dependent inhibition of CYP2C19-mediated S-mephenytoin 4’-hydroxylation, NBPB was preincubated with human liver microsomes in the presence of an NADPH-regenerating system. NBPB was found not to be a time-dependent inhibitor (data not shown).

The effect of NBPB on marker enzyme activities for CYP1A2 (phenacetin O-deethylation), CYP2A6 (coumarin 7-hydroxylation), CYP2B6 (bupropion hydroxylation), CYP2D6 (bufuralol 1’-hydroxylation), and CYP3A4 (testosterone 6β-hydroxylation) also were evaluated in human liver microsomes. Figure 1 illustrates that inhibition of these reactions caused by 0.25 and 6.25 µM NBPB was minimal. In fact, NBPB had very little effect on CYP1A2-, CYP2A6-, CYP2B6-, CYP2D6-, and CYP3A4-mediated reactions with IC₅₀ values >100, >100, 62, >100, and 89 µM, respectively (Table 1).

Discussion

In previous studies in our laboratory, the specificities of several isof orm-selective chemical inhibitors on CYP1A2-, CYP2C9-,
CYP2D6- and CYP3A4-mediated reactions in human liver microsomes were evaluated (Newton et al., 1995). At that time, no isoform-selective inhibitor for CYP2C19 had been reported in the literature. Many in vitro phenotyping studies have used omeprazole and transylcypramine as chemical inhibitors for CYP2C19, but CYP3A4/ CYP2C9 and CYP2A6 activities are also inhibited by omeprazole and transylcypramine, respectively (Ko et al., 1997; Zhang et al., 2001). Tucker et al. (2001) recommended ticlopidine and nootkatone as “acceptable” in vitro probe inhibitors for CYP2C19, although there were no “preferred” CYP2C19 inhibitors listed in that report or in the recent Pharmacetical Research and Manufacturers of America position papers (Bjornsson et al., 2003a,b). Ticlopidine was reported to be a selective mechanism-based inhibitor of CYP2C19 (Ha-Duong et al., 2000), but it is also a potent competitive inhibitor of CYP2C19 and CYP2D6 with Ki values of 1.2 and 3.4 μM, respectively (Ko et al., 2000). Nootkatone was not selective for CYP2C19 because it also showed a comparable level of inhibition on CYP2A6 activity in human liver microsomes (Tassaneeyakul et al., 2000). In fact, there were no known CYP2C19 selective inhibitors until (+)-N-3-benzyl-nirvanol and NBPP were synthesized and evaluated by Suzuki et al. (2002).

The high potency and selectivity of (+)-N-3-benzyl-nirvanol and NBPP should enable their effective use as CYP2C19 inhibitors for reaction phenotyping studies. One may argue that CYP2C19 isoform-specific antibodies may be used for reaction phenotyping. However, due to the high sequence homology between CYP2C enzymes, adequate specificity of an antibody often is difficult to achieve in such situations. For example, several inhibitory anti-CYP2C19 antibody preparations also slightly inhibited reactions catalyzed by other isoforms of the CYP2C subfamily (Lasker et al., 1998; Schulz-Utermoehl et al., 2000; Wester et al., 2000). The monoclonal antibody (mAb 1-7-4-8) generated by Krausz et al. (2001) seems to be effective, as it was reported to be specific and potent for CYP2C19. Since the availability and high cost of the antibody may limit its utility, a less expensive and more readily available isoform-selective chemical inhibitor would be a very useful alternative for P450 isoform identification. However, when a chemical inhibitor is used for in vitro reaction-phenotyping studies, its selectivity for a given P450 isofrom has to be carefully evaluated. In addition, to ensure the inhibitory potency, relatively high concentrations of the inhibitor (K i \approx 10; \text{Ki} \leq 10) are used to achieve a high degree of inhibition (Rodrigues, 1999).

In the present study, the potency and selectivity of NBPP as a CYP2C19 inhibitor in human liver microsomes were clearly demonstrated. Therefore, this compound can be used to establish the relative contribution of CYP2C19 toward the total metabolism of therapeutic agents in human liver microsomes. The compound also may serve as a positive control for routine reversible inhibition screening of CYP2C19-mediated reactions when human liver microsomes are used as the enzyme source.

### References


Tassaneeyakul W, Guo L, Fukuda K, Ohta T, and Yamazoe Y (2000) Inhibition selectivity of known CYP2C19 selective inhibitors listed in that report or in the recent Pharmacetical Research and Manufacturers of America position papers (Bjornsson et al., 2003a,b). Ticlopidine was reported to be a selective mechanism-based inhibitor of CYP2C19 (Ha-Duong et al., 2000), but it is also a potent competitive inhibitor of CYP2C19 and CYP2D6 with Ki values of 1.2 and 3.4 μM, respectively (Ko et al., 2000). Nootkatone was not selective for CYP2C19 because it also showed a comparable level of inhibition on CYP2A6 activity in human liver microsomes (Tassaneeyakul et al., 2000). In fact, there were no known CYP2C19 selective inhibitors until (+)-N-3-benzyl-nirvanol and NBPP were synthesized and evaluated by Suzuki et al. (2002).

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