High-Affinity Binding of [3H]Cimetidine to a Heme-Containing Protein in Rat Brain

Rebecca Stadel, Jun Yang, Julia W. Nalwalk, James G. Phillips, and Lindsay B. Hough

Center for Neuropharmacology and Neuroscience, Albany Medical College, Albany, New York (R.S., J.Y., J.W.N., L.B.H.); and Curragh Chemicals, Cleveland, Ohio (J.G.P.)

Received July 26, 2007; accepted December 18, 2007

ABSTRACT:

[3H]Cimetidine (3HCIM) specifically binds to an unidentified site in the rat brain. Because recently described ligands for this site have pharmacological activity, 3HCIM binding was characterized. 3HCIM binding was saturable, heat-labile, and distinct from the histamine H2 receptor. To test the hypothesis that 3HCIM binds to a cytochrome P450 (P450) (P450), the effects of nonselective and isoform-selective P450 inhibitors were studied. The heme inhibitor KCN and the nonselective P450 inhibitor metyrapone both produced complete, concentration-dependent inhibition of 3HCIM binding (Kᵢ = 1.3 mM and 11.9 μM, respectively). Binding was largely unaffected by inhibitors of CYP1A2, 2B6, 2C8, 2C9, 2D6, 2E1, and 19A1 but was eliminated by inhibitors of CYP2C19 (tranylcypromine) and CYP3A4 (ketoconazole). Synthesis and testing of CC11 [4(5-((4-iodobenzyl)-thiomethyl)-1H-imidazole] and CC12 [4(5-((4-iodobenzyl)-thiomethyl)-1H-imidazole] confirmed both drugs to be high-affinity inhibitors of 3HCIM binding. On recombinant human P450s, CC12 was a potent inhibitor of CYP2B6 (IC₅₀ = 11.7 nM), CYP2C19 (51.4 nM), and CYP19A1 (140.7 nM) and had a range of activities (100–494 nM) on nine other isoforms. Although the 3HCIM binding site pharmacologically resembles some P450s, eight recombinant human P450s and three recombinant rat P450s did not exhibit 3HCIM binding. Inhibition by KCN and metyrapone suggests that 3HCIM binds to a heme-containing brain protein (possibly a P450). However, results with selective P450 inhibitors, recombinant P450 isoforms, and a P450 antibody did not identify a 3HCIM-binding P450 isoform. Finally, CC12 is a new, potent inhibitor of CYP2B6 and CYP2C19 that may be a valuable tool for P450 research.

Cimetidine (Fig. 1), a well known H₂ receptor antagonist, is used to reduce gastric acid secretion in the treatment of various gastrointestinal disorders. In general, H₂ antagonists are considered to be relatively safe drugs, with less than 3% of the population reporting side effects, most of which are minor (Goodman et al., 2001). In laboratory animals and/or humans, however, cimetidine can produce significant additional effects including mental confusion, seizures, antinociception, and adverse drug interactions (Goodman et al., 2001; Cannon et al., 2004). The antinociceptive and seizure-producing effects of cimetidine are actions on the brain that are not mediated by the H₂ receptor (Cannon et al., 2004). Adverse drug interactions with cimetidine have been attributed to its well known ability to act as a low-potency inhibitor of the cytochrome P450 (P450) superfamily (Sorkin and Darvey, 1983).

Brain membranes were reported to exhibit high-affinity binding of [3H]cimetidine (3HCIM) nearly 30 years ago (Burkard, 1978). Although early studies concluded that this binding represented brain H₂ receptors, subsequent work showed that this conclusion was incorrect. For example, imidazole-containing H₂ ligands have high affinity for the brain 3HCIM-binding site, but nonimidazole H₂ ligands lack such activity (Rising et al., 1980; Smith et al., 1980; Warrander et al., 1983). Furthermore, Warrander et al. (1983) reported the existence of imidazole-containing compounds with high affinity for the 3HCIM-binding site but lacking affinity for the H₂ receptor. Thus, cimetidine has a high-affinity, biological target in the brain that is distinct from the H₂ receptor and remains to be identified. Liver, kidney, and gastric mucosa have also been reported to contain a 3HCIM-binding site (Rising et al., 1980; Chansel et al., 1982; Reilly et al., 1983; Warrander et al., 1983).

Because several of the side effects of cimetidine are not mediated by H₂ receptors, the identity of the 3HCIM-binding site is of biological interest. For example, we recently suggested that the 3HCIM-binding site might represent an antinociceptive target. In support of this idea, CC12 (Fig. 1), a new derivative of cimetidine, showed high affinity for 3HCIM-binding sites and behaved in vivo as a competitive antagonist of several antinociceptive agents (Hough et al., 2007). In the present study, we have characterized the pharmacological nature of 3HCIM binding in brain homogenates and tested the hypothesis that this binding site is a member of the P450 superfamily.

Materials and Methods

Materials. ThioTEPA was purchased from Fisher Scientific Co. (Atlanta, GA). Quercetin, N,N-diethylthiocarbamate, and several P450s were purchased from Sigma-Aldrich (St. Louis, MO). Cimetidine and fluvoxamine were purchased from Tocris Bioscience (Ellisville, MO). We thank Prof. R. Leurs (Leiden Amsterdam Center for Drug Design, Amsterdam, Netherlands) for providing recombinant P450s.

ABBREVIATIONS: P450, cytochrome P450; 3HCIM, [3H]cimetidine; TEPA, N,N,N′-triethylene phosphoramide; CC11, 4(5)-((benzylthiomethyl)-1H-imidazole hydrochloride; CC12, 4(5)-((4-iodobenzylthiomethyl)-1H-imidazole; rP450, recombinant cytochrome P450; NADPH-RS, NADPH regenerating system; CO, carbon monoxide; NOS, nitric-oxide synthase.
formed following Smith et al. (1980). Resuspended crude membrane pellets suspended in assay buffer and assayed for 3HCIM-binding activity. For competition studies, relevant concentrations (IC50 values also not shown). Similar to previously published reports, the H2 receptor antagonists ranitidine (Smith et al., 1980) and zolantidine did not inhibit 3HCIM binding at H2-receptor relevant concentrations (IC50 values >30 μM, also not shown).

Pharmacological Characterization of the 3HCIM-Binding Site. Cimetidine is a well documented low-potency P450 inhibitor (Sorkin and Darvey, 1983). To investigate whether the 3HCIM-binding site resembles a P450-like protein, the effects of the nonselective P450 inhibitors metyrapone and cyanide were determined on 3HCIM binding (Fig. 3). Both drugs produced concentration-dependent inhibition [pIC50 values of −4.68 ± 0.04 (IC50 = 20.8 μM) and −2.65 ± 0.05 (IC50 = 2.2 mM), for metyrapone and cyanide, respectively].
Similar results were obtained from two other experiments.

Nonspecific binding was evaluated with 10 μM cimetidine, both giving the same results. Each sample was measured in triplicate, from two individual experiments (total of six replicates), with the ordinate representing the mean ± S.E.M. of triplicate determinations from a single experiment. Similar results were obtained from two other experiments.

![Figure 2](image1.png)  
**Figure 2.** Saturation of the 3HCIM-binding site in the rat brain. Whole brain crude membrane homogenates (390 μg) were incubated in triplicate with varying concentrations of 3HCIM (abscissa) for 60 min and then were filtered as described. Nonspecific binding was evaluated with 10 μM cimetidine. K_d and B_max values were estimated by nonlinear regression. Inset, the same data are shown in Scatchard format. Examples of the mean total and nonspecific binding, at 50 nM 3HCIM, were 5633 cpm (0.24 pmol) and 1588 cpm (0.07 pmol), respectively. The ordinate represents the mean ± S.E.M. of triplicate determinations from a single experiment.

![Figure 3](image2.png)  
**Figure 3.** Inhibition of 3HCIM binding by metyrapone and KCN. Whole brain crude membrane homogenates (360–470 μg of protein) were incubated in triplicate with 50 nM 3HCIM as in Fig. 2 with increasing concentrations of metyrapone and KCN (abscissa). Nonspecific binding was evaluated with 30 μM burimamide or 10 μM cimetidine, both giving the same results. Each sample was measured in triplicate, from two individual experiments (total of six replicates), with the ordinate representing the mean ± S.E.M. IC50 values were estimated by nonlinear regression (variable slope) and are provided.

A series of selective inhibitors of various P450 isoforms were chosen to further characterize 3HCIM binding (see Table 1 for inhibitors, P450 targets, and K_i values). Figure 4 shows the effects of these compounds on the 3HCIM-binding site. Fluvoxamine (an inhibitor of CYP1A2), thiotePA (CYP2B6), quercetin (CYP2C8), sulfaphenazole (CYP2C9), quinidine (CYP2D6), diethylthiocarbamate (CYP2E1), and n-aminoglutethimide (CYP19A1) all produced less than 50% inhibition of 3HCIM binding at 100 μM (Fig. 4), a concentration well above all of the reported P450-inhibitory potencies (Table 1). In contrast, ketoconazole (CYP3A4) and tranylcypromine (CYP2C19) both completely displaced 3HCIM binding with pIC50 values of 4.96 ± 0.15 (IC50 = 11.1 μM) and 4.72 ± 0.06 (IC50 = 18.9 μM) and Hill coefficients of −0.8 ± 0.2 and −0.8 ± 0.1, respectively (Fig. 4). The potency of ketoconazole as an inhibitor of 3HCIM binding (calculated K_i = 6.4 μM) (Table 1) is greater than 300-fold lower than the published affinity of this drug as a CYP3A4 inhibitor (K_i = 0.02 μM) (Table 1). Tranylcypromine, however, competed with 3HCIM binding at a concentration comparable with its potency as an inhibitor of CYP2C19 activity (K_i = 10.8 versus 8 μM, respectively) (Table 1). However, ticlopidine (another inhibitor of CYP2C19) had no effect on 3HCIM binding (Fig. 4).

**High-Affinity Ligands for 3HCIM-Binding Proteins.** The search for high-affinity ligands led to the study of the cimetidine analogs CC11 (Warrander et al., 1983) and CC12 (Hough et al., 2007) (Fig. 1). In agreement with earlier results (Warrander et al., 1983), CC11 produced complete, concentration-dependent inhibition of 3HCIM binding, yielding a pIC50 of 8.05 ± 0.03 (IC50 = 9.0 nM) and a Hill coefficient of −1.4 ± 0.1 (Fig. 5). CC12 was also shown to be a potent inhibitor of 3HCIM binding, producing a pIC50 of 7.78 ± 0.11 (IC50 = 16.5 nM) and a Hill coefficient of −1.1 ± 0.2 (Fig. 5).

Because CC12 is a potent inhibitor of 3HCIM binding, the effects of CC12 on several human P450 isoforms were measured in pilot studies. Five P450 isoforms previously reported to be inhibited by cimetidine (Table 2) were evaluated. Preliminary results indicated that CC12 produced concentration-dependent inhibition of all five enzymes, with estimated IC50 values between 51 and 494 nM (Table 2).

Due to the unexpectedly wide range of activities of CC12 and increased availability of commercial P450 testing, CC12 was subjected to preliminary screening on seven additional P450 human isoforms (Table 2). A single concentration of CC12 (200 nM) inhibited the activities of these enzymes as well (33–100%). Thus, CC12 exhibited some affinity for all 12 P450 isoforms.

Because of the apparently high affinity of CC12 for CYP2B6, CYP2C19, and CYP19A1 in these preliminary studies, additional detailed experiments were performed to validate these findings. CC12 produced concentration-dependent inhibition of CYP2B6, CYP2C19, and CYP19A1 activities, yielding pIC50 values of 7.93 ± 0.08 (IC50 = 11.7 nM), 7.29 ± 0.09 (IC50 = 51.4 nM), and 6.85 ± 0.06 (IC50 = 140.7 nM), respectively (Fig. 6).

**3HCIM-Binding Potential of Recombinant Candidate P450s.** Although low concentrations of CC12 bind to the 3HCIM-binding site and also inhibit the activities of CYP2B6, CYP2C19, and CYP19A1, only the selective CYP2C19 inhibitor, tranylcypromine, competed with 3HCIM binding at a concentration comparable with its potency as an inhibitor of CYP2C19 activity (K_i = 10.8 versus 8 μM, respectively) (Table 1). Therefore, studies similar to those in Fig. 7 showed that eight rat proteins (CYP2C11 and CYP2C6) as homologs of human CYP2C19 were sought. A National Center for Biotechnology Information BLAST search of the human 2C19 amino acid sequence was initiated to determine the relevance of CYP2C19, rat homologs of the human CYP2C19 were sought. A National Center for Biotechnology Information BLAST search of the human 2C19 amino acid sequence was performed against the rat genome. This BLAST search identified two rat proteins (CYP2C11 and CYP2C6) as homologs of human CYP2C19. Thus, experiments were performed to directly test the hypothesis that CYP2C11 or CYP2C6 accounts for the specific binding of 3HCIM in the rat brain.

When 3HCIM was incubated with sl9 microsomes containing rCYP2C11 (Fig. 7, left), no specific binding was measured. Companion studies were performed to ensure that the recombinant enzyme was viable and was properly filtered in the radioligand-binding assays (Fig. 7, right). To ensure that optimal binding conditions for the recombinant enzymes were met, variations in assay conditions, including adding higher enzyme amounts (4 pmol) or coincubating the enzyme (2–6 pmol/tube) with brain homogenate during radioligand-binding assays, were used and had no effect on binding activity. Furthermore, preincubation of the enzyme with 3HCIM and an NADPH-RS failed to produce a reliable specific radioligand binding signal. Additional studies similar to those in Fig. 7 showed that eight human P450s (2C19, 2A6, 2B6, 2C8, 2C9, 2C18, 3A5, and 19A1) and two other rat P450s (2C6 and 2B1, rat homologs of CYP2C19 and
CYP2B6, respectively) did not exhibit specific binding of 3HCIM (data not shown, see Table 2).

A CYP2C8/9/18/19 antibody was also used to further evaluate the similarities between 3HCIM binding in the rat brain and CYP2C19. As shown (Fig. 8), antibody treatment caused concentration-dependent inhibition of rCYP2C11 activity but had no effect on rat brain 3HCIM binding.

Discussion

In the present study we have confirmed that 3HCIM specifically binds to rat brain homogenates and have found that this binding is heat-labile, suggesting that it is proteinaceous in nature. The dissociation constant of the binding (K_D = 67 nM) (Fig. 2) is similar to several previously reported values [42 nM (Burkard, 1978), 52.2 nM (Rising et al., 1980), and 40 nM (Devoto et al., 1980)]. Additional K_D values from 15.7 to 400 nM (as reviewed in Warrander et al., 1983) have been reported. As discussed by Warrander et al. (1983), variations in assay conditions (including incubation temperature and time, buffering conditions, concentrations of radioligand studied, type of drug added to evaluate nonspecific binding, and species) may contribute to this variability. Such variations in conditions may also affect the measurement of receptor density. The B_max value currently reported (0.94 pmol/mg of protein) (Fig. 2) is consistent with several other laboratory results [0.68 (Rising et al., 1980) and 1.3 pmol/mg (calculated from Burkard, 1978)]. However, other reported values vary from 0.23 to 3.9 pmol/mg (Warrander et al., 1983). Compared with many receptors for biogenic amines in the brain, this density is high, yet higher levels have been found for the cannabinoid CB1 receptor in the brain (1.85 pmol/mg) (Devane et al., 1988). The high density of 3HCIM-binding sites (much greater than H2 receptor expression levels) (Gajtkowski et al., 1983) reinforces the conclusion that these sites are not the H2 receptor. The present findings, showing that pharmacologically active concentrations of ranitidine and zolantidine (both nonimidazole-containing H2 receptor antagonists) fail to inhibit 3HCIM binding, are similar to earlier results (Smith et al., 1980; Chansel et al., 1982; Gajtkowski et al., 1983; Warrander et al., 1983), further supporting the conclusion that the binding is not to the histamine H2 receptor. Furthermore, other histamine receptors or histamine-binding sites seem to be excluded by the finding that very high concentrations of histamine are required to inhibit 3HCIM binding (IC50 values were 0.8 mM in unpublished data from this laboratory and >1 mM from Smith et al., 1980).

Cimetidine is a well known inhibitor of several human P450 isoforms, although affinities are low (K_i values = 14–140 μM) (Table 1). These K_i values are much higher than the dissociation constant of cimetidine for the 3HCIM-binding site in the rat brain (K_D = 67 nM) (Fig. 2). Despite this discrepancy, it seems possible that 3HCIM could bind to a P450 at concentrations lower than those required to inhibit enzyme function. The K_i values for cimetidine on human P450s are
isoforms. The first approach was to study the effects of selective P450 inhibitors, two additional P450 isoforms (currently 88 P450 isoforms are known in the rat) of the lack of selectivity of these inhibitors and the multiplicity of consistent with (but not proof of) a P450-binding mechanism. Because cyanide and metyrapone (Fig. 3) (discussed further below) is also much higher than therapeutic plasma levels of cimetidine, even though these levels can modify drug metabolism (Madeira et al., 2004). This finding suggests the possibility that cimetidine may bind to additional P450 isoforms.

The existence of several cimetidine-P450 interactions seems to also much higher than therapeutic plasma levels of cimetidine, even though these levels can modify drug metabolism (Madeira et al., 2004). This finding suggests the possibility that cimetidine may bind to additional P450 isoforms.

The existence of several cimetidine-P450 interactions seems to support the hypothesis that the brain 3HCIM-binding site is a P450, even though this has never been tested. Inhibition of the binding by cyanide and metyrapone (Fig. 3) (discussed further below) is also consistent with (but not proof of) a P450-binding mechanism. Because of the lack of selectivity of these inhibitors and the multiplicity of P450 isoforms (currently 88 P450 isoforms are known in the rat) (http://drnelson.utmem.edu/CytochromeP450.html), two additional approaches were taken to identify candidate 3HCIM-binding P450 isoforms. The first approach was to study the effects of selective P450 inhibitors on 3HCIM binding (Fig. 4; Table 1). By initially focusing on P450 isoforms known to be inhibited by cimetidine (Table 1), it was predicted that if the 3HCIM-binding site resembles any of these enzymes should compete with 3HCIM binding at concentrations equivalent to (or less than) those reported to inhibit P450 activities.

### Table 2

<table>
<thead>
<tr>
<th>P450 Isoform</th>
<th>Species</th>
<th>% Inhibition at 200 nM* or CC12 IC50 (μM)*</th>
<th>Cimetidine Ki (μM)*</th>
<th>Cimetidine Reference*</th>
<th>Tested for 3HCIM Binding**</th>
</tr>
</thead>
<tbody>
<tr>
<td>2B6</td>
<td>Human</td>
<td>100%** (0.0117)**</td>
<td>14</td>
<td>Cohen et al., 2003</td>
<td>Yes</td>
</tr>
<tr>
<td>2C9</td>
<td>Human</td>
<td>0.128a</td>
<td>140</td>
<td>Miners et al., 1988</td>
<td>Yes</td>
</tr>
<tr>
<td>3A5</td>
<td>Human</td>
<td>64%a</td>
<td>82</td>
<td>Kerlan et al., 1992</td>
<td>No</td>
</tr>
<tr>
<td>2A6</td>
<td>Human</td>
<td>62%a</td>
<td>None</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1A2</td>
<td>Human</td>
<td>0.120a</td>
<td>38</td>
<td>Madeira et al., 2004</td>
<td>No</td>
</tr>
<tr>
<td>2C10</td>
<td>Human</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2C11</td>
<td>Rat</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2C15</td>
<td>Rat</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2B1</td>
<td>Rat</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*IC50 values were estimated by nonlinear regression from pilot studies with three concentrations of CC12 in duplicate.

**Ki values for cimetidine taken from the literature cited.

---

FIG. 6. CC12 inhibition of CYP2B6, CYP2C19, and CYP19A1 activity. sf9 microsomes expressing rCYP2B6 (2 pmol), rCYP2C19 (2 pmol), or rCYP19A1 (0.4 pmol) were assayed in the presence of varying concentrations of CC12 (abscissa) as described. Enzymatic activity (ordinate) is reported as the mean (± S.E.M.) activity from three separate experiments, each determined in triplicate (or in one case duplicate). Basal activities (mean ± S.E.M., in the absence of competing ligand) were 1.362 ± 0.050, 0.296 ± 0.021, and 0.016 ± 0.001 nmol/(min × mg of protein) for CYP2B6, CYP2C19, and CYP19A1, respectively. Estimated IC50 values for CC12 on each enzyme are given.

FIG. 7. Lack of 3HCIM specific binding to rCYP2C11. Left, sf9 microsomes expressing CYP2C11 (0.5 or 2.0 pmol of enzyme (1.35 or 5.4 μg of protein, respectively) per tube) or nontransfected microsomes (control, 10 μg protein) were incubated with 3HCIM (50 nM) and filtered as in Fig. 3. 3HCIM binding (left ordinate, mean ± S.E.M. from triplicate determinations) is shown from a single experiment. Right, to ensure that CYP2C11 was properly filtered by the binding assay, CYP2C11 (50 pmol of enzyme, 135 μg of protein) or liver membranes (100,000 g pellet, 387 μg) were incubated as in the left panel but in the absence of 3HCIM. Samples were filtered with either filter paper present or absent, followed by washing as described, or not filtered at all (100% controls). Filters or control samples (860 μl) were analyzed for CYP2C11 activity as described. Control enzyme activities of the unfiltered CYP2C11 sample (13.5 μg of protein) and the unfiltered liver microsome sample (38.2 μg of protein) were 5.7 and 1.3 nmol/ (min × mg of protein), respectively. The right ordinate shows percent control enzyme activity detected (mean ± S.E.M.) in two to three determinations from one experiment. These experiments show that CYP2C11 is viable (right), is filtered in the radioligand-binding assay (right), but does not specifically bind 3HCIM (left). NS, nonspecific binding.
The findings that pharmacologically active concentrations of fluvoxamine, sulfaphenazole, quinidine, and diethylthiocarbamate all failed to inhibit 3HCIM binding (Fig. 4; Table 1) suggest that the 3HCIM-binding site does not resemble human CYP1A2, 2C9, 2D6, or 2E1, respectively. Moreover, the binding site does not resemble CYP3A4, as the 3HCIM-binding constant of ketocazole (Kᵢ = 6.4 μM) (Fig. 4; Table 1) is much larger than the comparable value on CYP3A4 enzyme activity (Kᵢ = 0.02 μM). In contrast, the affinity of tranylcypromine for the binding site (Kᵢ = 10.8 μM) is virtually identical with its activity at CYP2C19 (Kᵢ = 8 μM) (Fig. 4; Table 1), suggesting that the 3HCIM-binding site may resemble a CYP2C19-like protein. This similarity was not sustained by results with other CYP2C19 inhibitors, as ticlopidine was inactive on the binding site (Fig. 4; Table 1). Because ticlopidine has been suggested to be a mechanism-based suicide inhibitor of CYP2C19 (Ha-Duong et al., 2001), additional experiments (i.e., preincubation of brain membranes with ticlopidine at 37°C with NADPH-RS) ensured that this drug did not require metabolic activation to inhibit 3HCIM binding (data not shown).

Because pharmacological screening failed to clearly implicate a 3HCIM-binding candidate P450, other targets for tranylcypromine and ketoconazole were considered. Although these drugs are widely used as isofom-selective inhibitors (Charet et al., 1997; Erickson et al., 1999), both have documented actions at additional P450 isoforms. In addition to its activity on CYP2C19, tranylcypromine has been reported to inhibit CYP2B6 and CYP19A1 (Stresser, 2004), but failure of thioTEPA (an inhibitor of 2B6) and DL-aminoglutethimide (an inhibitor of 19A1) to displace 3HCIM binding (Fig. 4; Table 1) indicates that the binding site does not resemble these enzymes. Tranylcypromine is also a potent inhibitor of CYP2A6 (IC₅₀ = 0.05 μM) (Draper et al., 1997), but the binding site seems to not resemble this isoform because the affinity of tranylcypromine for 3HCIM binding is much lower (IC₅₀ = 10.8 μM) (Table 1). Ketoconazole is a well known inhibitor of CYP3A4, but this drug has also been reported to inhibit CYP2C8 at concentrations (IC₅₀ = 4.4 μM) (Stresser, 2004) similar to its observed affinity for 3HCIM binding (IC₅₀ = 11.1 μM) (Fig. 4; Table 1), suggesting the possible relevance of this isoform. However, the inability of quercetin (another 2C8 inhibitor) to influence 3HCIM binding (Fig. 4; Table 1) seems to exclude a role for CYP2C8-like activity. Thus, despite these documented additional actions of tranylcypromine and ketoconazole, no clear 3HCIM-binding P450 candidate emerged from the pharmacological screening.

High-affinity ligands were synthesized and tested on 3HCIM-binding and P450 activities as a second approach toward identifying 3HCIM-binding proteins. Warrander et al. (1983) showed that the 3HCIM-binding site was not the H₂ receptor by demonstrating a series of cimetidine analogs that competed with 3HCIM binding but lacked H₂ receptor activity. One compound (herein named CC11) from this study had high affinity for the binding site (Kᵢ = 5 nM) (Warrander et al., 1983), but the synthesis, rationale, and possible relevance to P450s were not given. CC11 was synthesized and tested in this study presently to confirm the high affinity of this drug for 3HCIM binding (Kᵢ = 5.2 nM) (Fig. 5). Although not mentioned by Warrander et al. (1983), earlier literature reported CC11 to be a weak inhibitor of aldrin epoxidation (a P450-mediated reaction) in rat liver microsomes (Wilkinson et al., 1972).

CC12 (the para-iodo derivative of CC11) (Fig. 1) was described for the first time in 2007 (Hough et al., 2007), and the activity of this drug on P450 enzymes has not been previously studied. Originally synthesized to investigate the biological relevance of 3HCIM binding, the compound is a potent ligand for the binding site (Kᵢ = 9.5 nM) (as in Fig. 5) and also behaves in vivo as an antagonist of several analgesic drugs including morphine and the cimetidine derivative imiprogan (Hough et al., 2007). The mechanisms accounting for this latter activity are not known (Hough et al., 2007). CC12 is found to be a moderately potent H₂ antagonist (Kᵢ = 50 nM), but the drug lacks activity in vitro on a variety of other central nervous system receptors (Hough et al., 2007). Chemically, CC12 belongs to the family of phenylimidazole P450 inhibitors that commonly interact with the heme moiety of P450, yielding a “type II” difference spectrum (Correia and Ortiz de Montellano, 2005). It is remarkable that within this family, the nature of the aromatic heteroatom and the structure of the side chain still permit a wide variety of potencies and selectivities for various isoforms. Drugs from this class include metyrapone, ketoconazole, and letrozole (Correia and Ortiz de Montellano, 2005). However, even when compared with many of these other compounds, CC12 is a very high-potency P450 inhibitor (on CYP2B6, IC₅₀ = 11.7 nM, equivalent to Kᵢ = 5.4 nM) (Draper et al., 2000). Other compounds reported to act on CYP2B isoforms include miconazole (Kᵢ = 50 nM) (Zhang et al., 2002), sulconazole (Kᵢ = 40 nM) (Zhang et al., 2002), and 4-4-chlorophenyl)imidazole (IC₅₀ = 70 nM) (Spatzzenegger et al., 2001). Additional studies are needed to assess the potency, selectivity, and utility of CC12 as a tool for P450 research.

Because CC12 has high affinity for the 3HCIM-binding site (Kᵢ = 9.5 nM) (Fig. 5), this drug should be a potent inhibitor of any P450 isoform capable of 3HCIM binding. Although CC12 showed inhibitory activity on a total of 12 P450s (estimated IC₅₀ values = 11.7–494 nM) (Table 2; Fig. 6), this drug showed high affinity for only three isoforms: CYP2B6, CYP2C19, and CYP19A1 (IC₅₀ values = 11.7, 51.4, and 140.7 nM, respectively) (Fig. 6). The lack of 3HCIM-binding affinity by inhibitors of CYP2B6 and CYP19A1 (Fig. 4; Table 1) suggests little relevance of these isoforms to the binding site. However, two inhibitors of CYP2C19 (CC12 and tranylcypromine) also inhibited 3HCIM binding, whereas another inhibitor (ticlopidine) did not, suggesting the possible significance of CYP2C19-like proteins.
indicates that these proteins (which are highly homologous to human CYP2C19) do not account for 3HCIM binding in the rat brain. Furthermore, the findings that rCYP2A6, 2B6, 2C8, 2C9, 2C18, 2C19, 3A5, and 19A1, as well as the 2B6 rat homolog, rCYP2B1, all failed to bind 3HCIM (Table 2) suggest that these enzymes do not resemble 3HCIM-binding proteins.

The conclusion that the rat brain 3HCIM-binding site does not resemble any of the P450s presently tested assumes that these methods would have detected 3HCIM binding to the rP450s. Calculations show that the amount of enzymes added to the binding assay (2 pmol) should have yielded a specific binding signal of 0.85 pmol (43% of the P450s should have bound according to a Keq of 67 nM) (Fig. 2). Such a result would have been easily detectable, because it corresponds to a signal approximately twice that of the Bmax value for rat brain homogenate (Fig. 2). It was also necessary to show that the P450 proteins, supplied in insect microsomal fractions, could be filtered in the radioligand binding assay. This result was confirmed by the lack of enzyme activity in the filtrate of the binding assay (Fig. 7, right).

The conclusion that the binding brain site does not resemble any of the currently tested P450s could also depend on specific differences in rat brain and insect cell preparations. For example, the recombinant enzyme systems currently studied are coexpressed with high levels of cytochrome P450 reductase and cytochrome b5. If these proteins prevented 3HCIM binding, then coinoculation of the insect microsomes with brain homogenates should have decreased 3HCIM binding in the homogenates. Alternatively, if the recombinant enzymes (in the presence of an NADPH-RS) did not contain the necessary components and/or cofactors to bind 3HCIM (as might be present in brain homogenates), then coinoculation of the homogenate with the recombinant enzymes should have increased binding activity. Because neither of these results was obtained, it is concluded that differences in the brain and insect cell preparations are not relevant.

Other potential differences between the rat brain homogenate and the rP450s that might affect 3HCIM binding include posttranslational modifications and potential allelic variations of P450s. Although the potential significance of these differences is difficult to assess, the present use of a CYP2C8/9/18/19 antibody known to inhibit CYP2C11 activity (Park et al., 1989) and that is immunoreactive with 2C6 (Waxman et al., 1987) provided another approach. The finding that this antibody (at concentrations that inhibited rCYP2C11 activity) (Fig. 8) did not alter 3HCIM-binding activity in rat brain fractions reinforces the conclusion that the 3HCIM-binding site is not CYP2C6 or CYP2C11.

The potency of cimetidine as a P450 inhibitor has been reported to be increased after preincubation of the drug with rat liver microsomes and an NADPH-RS (Levine et al., 1998). Under these conditions, it has been suggested that the higher potency of cimetidine can be explained by the formation of a metabolite-intermediate complex with P450 isoforms (Levine et al., 1998). If this hypothesis is correct, then one reason why 3HCIM binding to rP450s was not detected could be that conditions for forming this complex were present in rat brain homogenates but not in insect cell microsomes. Accordingly, mimicking these preincubation conditions should have resulted in an increase in 3HCIM binding of rat homogenates, thereby permitting the detection of specific 3HCIM binding to rP450s. The finding that the NADPH-RS preincubation had no effect on 3HCIM binding suggests that metabolic activation is not required for 3HCIM binding in either the brain or in the recombinant systems.

The present results show that the 3HCIM-binding site in rat brain is not CYP2C11, CYP2C6, or CYP2B1, but other P450 isoforms, and even non-P450 proteins remain to be tested. The present search for 3HCIM-binding P450s in the rat has depended heavily on testing the commercially available human isoforms. Rat P450s with high (>70%) homology to CYP2C19 (CYP2C6 and CYP2C11) lacked 3HCIM-binding activity. However, three additional rat P450s (CYP2C7, CYP2C24, and CYP2C79) also have high homologies to CYP2C9 (http://drnelson.utmem.edu/CytochromeP450.html) and remain viable candidates for 3HCIM-binding activity. None of these are commercially available, and one of these (CYP2C79) has only been described in silico. It is not known whether the CYP2C9/8/19/19 antibody used in the present study (Fig. 8) cross-reacts with these additional 2C isoforms. These isoforms remain to be cloned and tested for 3HCIM-binding activity.

Another limitation of the present study rests on the assumption that rat P450s with high homologies to a human P450 will share ligand-binding profiles. However, P450s are considered to be rather promiscuous, in that multiple enzymes from different families and subfamilies, can have overlapping substrate/inhibitor profiles (Rendic, 2002). For example, mutations of only three amino acid residues can alter CYP2C19 substrate selectivity to more closely resemble that of CYP2C9 (Jung et al., 1998). Thus, it is possible that the only homologies relevant to 3HCIM and CC12 binding are those in the substrate and inhibitor recognition sites. Subtle rat-human differences might also explain why the rat-binding protein shows 2C19-like activity with some, but not all, 2C19 inhibitors. Because crystal structures and homology models of several P450s with inhibitors have been described (Otyepka et al., 2007), it may be possible to use this approach in future searches for 3HCIM-binding P450s.

The classic demonstration of the existence of a P450 in tissues or of a drug-P450 interaction is the carbon monoxide (CO) difference spectrum, in which dithionite-reduced microsomes combine with CO to yield the characteristic 450-nm spectrum (Guengerich, 1991). Thus, the ideal experiment to demonstrate high-affinity binding of cimetidine to brain P450s would use cimetidine-induced alterations in the CO difference spectra from brain. Unfortunately, brain P450s are not in high enough abundance to yield a reproducible CO difference spectrum (X. Ding, personal communication).

If the 3HCIM-binding protein in brain is not a P450, then inhibition of this binding by cyanide (Fig. 3) suggests the importance of other heme-containing proteins. Cyanide is a well known inhibitor of P450s with affinities similar to values found in the present study (IC50 values = 1–2 mM) (Kitada et al., 1977). However, this reagent also inactivates other iron-containing (especially heme-containing) non-P450 proteins (Dixon and Webb, 1964). A query of the rat proteome (UniProt Knowledgebase) using the Integr8 Web Portal (http://www.ebi.ac.uk/integr8/Integr8-HomePage.do) with the gene ontology parameter “heme-binding” (GO:0020037) returns 115 unique heme-binding proteins. Approximately one-half of these are P450s. Notable among the remaining half are several electron transport proteins (e.g., cytochrome-related), oxygen transporters (hemoglobin, myoglobin, cytochrome, and neuroglobin), and other oxidases and peroxidases. The list also includes specialized enzymes such as soluble guanylate cyclase and three forms of nitric-oxide synthase (NOS). Some of these hemoproteins can be excluded as potential 3HCIM-binding targets because they are soluble and would not have been present in the membrane fractions used in the present study. Two other metalloenzymes (endothelial NOS and neural NOS) are unlikely to bind 3HCIM because CC12 lacked activity on these enzymes (Hough et al., 2007). In contrast to the case with cyanide, a literature review of metyrapone shows that its experimental use has been limited to P450 inhibition (Ignarro et al., 1985; Masubuchi et al., 1998). Thus, inhibition of 3HCIM binding by metyrapone suggests that the binding site may be a rat P450 isoform that was not tested in the present study. Recently we used the high affinity of CO for heme in unpublished pilot studies and found that 3HCIM binding in brain homogenates was reduced by
approximately 30% after CO treatment under reducing (dithionite) conditions. These findings, along with cyanide results, suggest the relevance of heme-containing proteins (P450 or non-P450).

The present studies suggest that the 3HCIM-binding site in brain is a heme-containing protein, but its identity as a P450 could not be confirmed. This protein needs to be identified for several reasons, including the biological activity of inhibitors of this binding (e.g., CC12), the pharmacological activities of cimetidine that are not mediated by the H2 receptor and the well known (but poorly understood) nature of cimetidine-P450 drug interactions. Furthermore, the affinity of cimetidine for this binding site is more than 10-fold higher than the affinity for any other known protein (including the H2 receptor) (Gajtkowski et al., 1983), arguing for its potential importance. Several approaches may be needed to identify this site, including cloning and testing of additional rat P450 isoforms and non-P450 hemoproteins, modeling ligand-P450 active sites or protein purification.

Acknowledgments. We thank Amanda B. Carpenter and Konstan-tina Svokos for excellent technical assistance. We also thank Dr. Melissa VanAlstine for discussions and pilot results. Dr. Mark Fleck and Dr. Xinxin Ding provided helpful comments on the manuscript and the project.

References


Address correspondence to: Dr. Lindsay B. Hough, Center for Neuropharmacology and Neuroscience, Albany Medical College-MC-136, 47 New Scotland Ave, Albany, NY 12208. E-mail: houghlb@amc.edu