MICROSOMAL PROTEIN CONCENTRATION MODIFIES THE APPARENT INHIBITORY POTENCY OF CYP3A INHIBITORS

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ABSTRACT:
The effect of microsomal protein concentration on the inhibitory potency of a series of CYP3A inhibitors was assessed in vitro using diazepam 3-hydroxylation (yielding temazepam) as an index of CYP3A activity. With diazepam concentrations fixed at 100 μM, inhibition of temazepam formation by fixed concentrations of ritonavir, ketoconazole, itraconazole, OH-itraconazole, norfluoxetine, and fluvoxamine decreased substantially as active protein concentrations increased from 0.0625 to 3.0 mg/ml. However protein concentration had only a small effect on the inhibitory activity of fluconazole. Equilibrium dialysis indicated extensive microsomal binding of all inhibitors except fluconazole; binding increased with higher protein concentrations. Based on the CYP3A content of liver microsomes, decrements in inhibitory potency of stronger inhibitors (ketoconazole and ritonavir) could be explained by specific binding, whereas nonspecific binding is anticipated to account for the effect on weaker inhibitors (norfluoxetine and fluvoxamine). Thus, microsomal binding (specific, nonspecific, or a combination of both) may have a major effect on estimation of inhibitory potency of P450 inhibitors and may contribute to variations among laboratories. The effect can be minimized by use of the lowest possible microsomal protein concentration for in vitro studies of metabolic inhibition.

Microsomal binding of drug substrates and/or metabolic inhibitors is increasingly recognized as a potential source of artifact arising in the course of in vitro studies of drug metabolism. Nonspecific binding of substrate to microsomal protein can influence availability of the substrate to the metabolizing enzyme or enzymes in vitro, and thereby yield biased estimates of enzyme kinetic parameters. These, in turn, may produce inaccurate predictions when in vitro data are used to estimate in vivo pharmacokinetics (Obach, 1996, 1997; Obach et al., 1997; McLure et al., 2000; Venkatakrishnan et al., 2000a, 2001; Kalvass et al., 2001). Inhibitor binding to microsomal systems may likewise influence estimation of potency of metabolic inhibitors (Gibbs et al., 1999a). The influence of binding has been termed “inhibitor depletion” when the inhibitor interacts with the active site (Gibbs et al., 1999a). However, inhibitor depletion could also refer to nonspecific inhibitor binding to microsomal preparations, as well as to actual microsomal consumption of the inhibitor itself through bio-transformation.

The present study evaluated the influence of microsomal protein concentration on the inhibitory capacity of known CYP3A inhibitors. In vitro 3-hydroxylation of diazepam to temazepam was used as an index reaction to profile CYP3A activity. Two selective serotonin reuptake inhibitors (fluvoxamine and norfluoxetine), three antifungal azole agents (itraconazole, ketoconazole, and fluconazole), a metabolite of itraconazole (OH-itraconazole), and the human immunodeficiency virus protease inhibitor ritonavir were used as representative CYP3A inhibitors. These agents have different intrinsic inhibitory capacities and different binding affinities.

Materials and Methods

Incubation Procedures and Index Reaction Characteristics. Liver samples from individual human donors with no known liver disease were provided by the International Institute for the Advancement of Medicine (Exton, PA), the Liver Tissue Procurement and Distribution System (University of Minnesota, Minneapolis, MN), or the National Disease Research Interchange (Philadelphia, PA). All samples were of the CYP2D6 and CYP2C19 metabolizer phenotype based on prior in vitro phenotyping studies. Microsomes were prepared by ultracentrifugation; microsomal pellets were suspended in 0.1 M potassium phosphate buffer containing 20% glycerol and stored at −80°C until use. Chemical reagents and drug entities were purchased from commercial sources or kindly provided by their pharmaceutical manufacturers (von Moltké et al., 1993, 1994a,b, 1996a,b; Venkatakrishnan et al., 2000a,b,c, 2001a,b).

Diazepam 3-hydroxylation to form temazepam was used as the index reaction for CYP3A activity (Andersson et al., 1994; Uno et al., 1996; Jung et al., 1997; Venkatakrishnan et al., 2000). Methanolic solutions of diazepam (100 μM) were added to 2-ml microcentrifuge tubes, and the solvent was evaporated to dryness under conditions of mild vacuum at 40°C. Incubation mixtures containing an isocitrate/isocitric dehydrogenase regenerating system in 50 mM phosphate buffer, 5 mM Mg2+, and 0.5 mM NADP+ were then added to the tubes and equilibrated in a 37°C water bath for 5 min.

Metabolic Inhibitors. Incubations were performed with diazepam alone (no inhibitor), and with coaddition of fixed concentrations of the following established CYP3A inhibitors: ketoconazole, 0.05 μM; itraconazole, 1.0 μM; OH-itraconazole, 0.25 μM; fluconazole, 10 μM; ritonavir, 0.05 μM; norfluoxetine, 25 μM; and fluvoxamine, 50 μM (von Moltké et al., 1994b, 1995, 1996a,b, 1998a,b; Venkatakrishnan et al., 2000b).
Influence of Active Protein Concentration. Reactions were initiated by addition of varying amounts of microsomal protein (0.0625, 0.125, 0.25, 0.5, 0.75, 1, 2, and 3 mg/ml) with final volume of 250 μl and incubated for 10 min. Previous studies had demonstrated time-dependent linearity of temazepam formation up to 20 min. After the incubation period, 100 μl of 2% butylated hydroxytoluene in acetonitrile was added to stop the reactions. A benzoazepine analog (U31485, 100 ng) (Greenblatt et al., 1981) was added as internal standard. Samples were then extracted by addition of 1.2 ml of toluene/isoamyl alcohol (98:5:1.5), vortexing vigorously for approximately 60 s, and centrifuging at 3000 rpm for 10 min. The organic phase was then transferred into 2-ml autosampling vials for gas chromatographic (GC) analysis. All reactions were performed in duplicate, and all studies were performed using microsomal preparations from four different human livers.

Influence of Inactive Protein Concentration. Diazepam (100 μM) with or without ketoconazole (0.05 μM), OH-itraconazole (0.25 μM), or fluconazole (10 μM) was added into 2-ml microcentrifuge tubes and dried under mild vacuum at 40°C. Buffer containing isocitrate/isocitric dehydrogenase system (as described above) was added and the tubes were incubated at 37°C for 5 min. Reactions were initiated by addition of varying amounts of heat-inactivated microsomal protein (0.0, 0.25, 0.75, 1, 1.75, and 2.75 mg/ml) with a constant amount of active microsomal protein (0.25 mg/ml). The final volume was 250 μl. After 10 min of incubation, 100 μl of 2% butylated hydroxytoluene in acetonitrile was added to stop the reactions. U31485 was added as internal standard, and samples were extracted with toluene/isoamyl alcohol for GC analysis as described above.

Analytical Conditions for Gas Chromatography. The GC (model 6890A; Hewlett Packard, Palo Alto, CA) was equipped with an electron-capture detector, automatic sampler, and data processor integrator. The column was 6 feet in length, 2 mm in internal diameter, and packed with 3% SP-2250 on Carbowax 20M (Supelco, Bellefonte, PA). The chromatographic conditions were oven temperature, 275°C; injection port temperature, 310°C; and detector temperature, 310°C. The carrier gas was argon/methane (95:5) at a flow rate of 30 ml/min. The retention times for diazepam (substrate), desmethyldiazepam (N-demethylated metabolite), temazepam (3-hydroxymethyl metabolite), and U31485 (internal standard) were 2.6, 3.7, 5.4, and 8.5 min, respectively.

Studies of Inhibitor Consumption. Ketoconazole (5 μM), with or without diazepam (100 μM), was added into 2-ml microcentrifuge tubes and dried under mild vacuum at 40°C. Reactions were initiated by addition of microsomal protein, and stopped by addition of 100 μl of acetonitrile. Terfenadine (15 μg) was added as internal standard. The samples were centrifuged at 14,000 rpm for 10 min. Twenty-five microliters of the supernatant was injected on the column for GC analysis of remaining ketoconazole...
where IC_{50} is the 50% inhibitory concentration for unbound inhibitor. For noncompetitive inhibition, IC_{50} = K_i, where K_i is the unbound inhibition constant. For competitive inhibition, IC_{50} = K_i (1 + S/K_m), where S is the concentration of substrate (diazepam, 100 μM), and K_m is the Michaelis constant for the conversion of diazepam into temazepam. The possibility that diazepam 3-hydroxylation may follow an atypical sigmoidal kinetic profile (Andersson et al., 1994) was not considered in this scheme.

I_{50} can be calculated from the added (free plus bound) inhibitor concentration (L) and the inhibitor free fraction (FF) in the microsomal mixture, as follows:

\[ I_{50} = I \cdot FF \]  

Using standard ligand binding relationships, FF can be expressed as follows:

\[ FF = \frac{1}{1 + K \cdot P} \]  

where P is the total concentration of available binding sites, and K is a constant representing the average affinity of the inhibitor for the binding sites. Combining eqs. 2, 3, and 4 yields

\[ R_c = \frac{I_{50}}{I_{50} + L/(1 + K \cdot P)} \]  

This equation was fitted to data points (P and R_c) using nonlinear regression to determine values of IC_{50} for each inhibitor. For equilibrium dialysis studies, free fraction was calculated as the ratio of inhibitor concentration in the dialysate divided by the concentration in the retentate.

Results

**CYP3A Inhibition Studies:** Effect of Active Protein Concentration. Temazepam formation in the absence of inhibitors increased as microsomal protein concentration increased to 3 mg/ml. This was accompanied by some degree of substrate consumption, based on GC analysis of diazepam remaining in the reaction mixture (Fig. 1). The inhibitory capacity of the various CYP3A inhibitors declined as microsomal protein concentration increased (Table 1). At low concentrations of microsomal protein (0.0625 mg/ml), inhibitors reduced temazepam formation rate to 23 to 49% of control. At the highest concentration of microsomal protein (3 mg/ml), these inhibitors reduced metabolite formation to no less than 80% of control velocity. The only exception was 10 μM fluconazole, for which inhibitory capacity was less dependent on protein concentration.

The relation of R_c to total microsomal protein concentration was consistent with eq. 5 for ritonavir (r^2 = 0.995), ketoconazole (r^2 = 0.979), fluvoxamine (r^2 = 0.928), norfluoxetine (r^2 = 0.968), itraconazole (r^2 = 0.959), and OH-itraconazole (r^2 = 0.982), but not for fluconazole (r^2 = 0.74) (Fig. 2). Estimated unbound IC_{50} values are shown in Table 1.

**Inhibitor Metabolism Studies.** No evidence of ketoconazole consumption was observed with increasing microsomal protein concentration. Ketoconazole concentrations remaining in incubates containing no microsomal protein were essentially identical to those in incubates containing protein, regardless of the actual protein concentration. The same relationship was obtained with coaddition of diazepam to the incubation mixtures.

**Inactive Protein Studies.** Temazepam formation in the presence of a constant active protein concentration (0.25 mg/ml) decreased with increasing concentrations heat-inactivated microsomal protein (0–2.75 mg/ml). The inhibitory capacity of 0.05 μM ketoconazole and 0.25 μM OH-itraconazole also declined with increasing inactive protein concentration (Table 2; Fig. 3); however, the effect was less pronounced than that in the active protein study. Fluconazole, on the other hand, showed little change in its inhibitory capacity with increasing inactive protein concentration.

**Equilibrium Dialysis Studies.** The free fraction of all inhibitors except fluconazole declined as the microsomal protein concentration increased (Fig. 4). The free fraction for fluconazole, however, was close to 1.0 regardless of microsomal protein concentration.

Discussion

Consistent with previous reports (Gibbs et al., 1999a), the CYP3A inhibitory effect of ketoconazole was substantially reduced with increasing concentrations of active microsomal protein. Because some substrate depletion also occurred at higher protein concentrations, the
effect of protein concentration on ketoconazole inhibition was, if anything, underestimated. Metabolic consumption of ketoconazole in the microsomal system was evaluated as a possible contributor to the decrement in inhibitory action of ketoconazole. Erve et al. (2000) reported substantial metabolic consumption of 1 μM ketoconazole by liver microsomes at a protein concentration of 2 mg/ml and an incubation duration of 30 min. However, we did not observe evidence of ketoconazole consumption by microsomes at 5 μM ketoconazole, protein concentrations up to 3 mg/ml, and an incubation duration of 10 min. In contrast to ketoconazole, inhibition by fluconazole was minimally dependent on microsomal protein concentration. The differences between ketoconazole and fluconazole apparently are explained by their differing affinity for nonspecific binding sites. Equilibrium dialysis demonstrated decreased ketoconazole free fraction with increasing microsomal protein concentration, whereas fluconazole free fraction was close to 1.0 regardless of microsomal protein concentration.

A number of other established CYP3A inhibitors, including theazole derivative itraconazole (and its principal metabolite), the selective serotonin reuptake inhibitors norfluoxetine and fluvoxamine, and the viral protease inhibitor ritonavir, exhibited decreases in inhibitory potency with increasing active microsomal protein in a manner similar to that observed with ketoconazole. All of these compounds are lipophilic agents with moderate-to-extensive binding to human plasma proteins, and equilibrium dialysis studies indicated that microsomal binding in vitro apparently explains the decrement in inhibitory activity with increasing microsomal protein. The magnitude and rank order of estimated unbound IC₅₀ values for these agents parallel previously reported data (Venkatakrishnan et al., 2000b, 2001b; von Moltke et al., 1994b, 1995, 1996a,b, 1998a,b). We did not evaluate actual inhibitor consumption by microsomes for these other inhibitors.

Previous studies using diazepam 3-hydroxylation as an index reaction have demonstrated the functional CYP3A content of liver samples used in the present study to be approximately 125 pmol/mg protein (Venkatakrishnan et al., 2001a). At 0.05 μM (50 pmol/ml) ketoconazole and 0.5 mg/ml active protein (63 pmol/ml CYP3A3), diazepam 3-hydroxylation activity was reduced to approximately 50% of control, implying occupancy by ketoconazole of a corresponding fraction of CYP3A binding sites (Table 1). Under the assumption of a single diazepam active site per molecule of CYP3A3, 32 pmol/ml ketoconazole is anticipated to be bound to active sites, with the remaining 18 pmol/ml either unbound or bound to other sites. At

![FIG. 2. Fractional decrement in reaction velocity relative to inhibitor-free control (y-axis) in relation to active microsomal protein concentration (x-axis) for representative CYP3A inhibitors. Data points are mean values from Table 1. For ritonavir, ketoconazole, and fluvoxamine, data points are consistent with eq. 5; lines represent functions of best fit, with unbound IC₅₀ values shown in Table 1. Data points for fluconazole were not consistent with eq. 5.]

![FIG. 3. Mean (± S.E.) fractional decrement in reaction velocity relative to inhibitor-free control (y-axis) in relation to inactive microsomal protein concentrations (x-axis). Active protein concentration was fixed at 0.25 mg/ml.]

### TABLE 2

<table>
<thead>
<tr>
<th>Inhibitor (µM)</th>
<th>0 µg/ml</th>
<th>0.25 µg/ml</th>
<th>0.75 µg/ml</th>
<th>1.75 µg/ml</th>
<th>2.75 µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ketoconazole (0.05)</td>
<td>0.38 ± 0.04</td>
<td>0.43 ± 0.06</td>
<td>0.51 ± 0.05</td>
<td>0.56 ± 0.04</td>
<td>0.67 ± 0.04</td>
</tr>
<tr>
<td>OH-Itraconazole (0.25)</td>
<td>0.33 ± 0.05</td>
<td>0.37 ± 0.06</td>
<td>0.43 ± 0.07</td>
<td>0.54 ± 0.04</td>
<td>0.51 ± 0.03</td>
</tr>
<tr>
<td>Fluconazole (10)</td>
<td>0.42 ± 0.04</td>
<td>0.44 ± 0.04</td>
<td>0.48 ± 0.05</td>
<td>0.47 ± 0.08</td>
<td>0.49 ± 0.08</td>
</tr>
</tbody>
</table>
higher concentrations of microsomal protein, available active binding sites would exceed the availability of ketoconazole in the in vitro mixture, and the decrement in inhibitory capacity could be explained mainly by specific microsomal binding. Similar estimations would hold for ritonavir. However, for the less potent inhibitors norfluoxetine and fluvoxamine, 50% inhibition is achieved at concentrations that greatly exceed the availability of specific binding sites, and the decrement in inhibition would be explained mainly by nonspecific binding.

The present study indicates that microsomal binding may have a major impact on estimation of inhibitory potency using in vitro systems. Decrements in inhibitory activity with increasing microsomal protein concentration may be observed with strong as well as weak inhibitors. The mechanism of binding may be either specific, nonspecific, or a combination of the two. It is likely that the decrease in inhibitory potency observed for relatively weaker inhibitors at high microsomal concentrations is attributable mainly to nonspecific binding. The findings suggest that large variability among laboratories in estimated inhibitory potency of a single inhibitor versus a single substrate (such as ketoconazole versus midazolam) could in part be explained by variations in protein and/or enzyme concentration (Gascon and Dayer, 1991; Hargreaves et al., 1994; Wrighton and Ring, 1996). In general, the impact of microsomal binding, both specific and nonspecific, may be minimized by use of the lowest possible microsomal concentration.

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