Comparative Use of Isolated Hepatocytes and Hepatic Microsomes for Cytochrome P450 Inhibition Studies: Transporter-Enzyme Interplay

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ABSTRACT:

Accurate assignment of the concentration of victim drug/inhibitor available at the enzyme active site, both in vivo and within an in vitro incubation, is an essential requirement in rationalizing and predicting drug-drug interactions. Inhibitor accumulation within the liver, whether as a result of active transport processes or intracellular binding, may best be accounted for using hepatocytes rather than hepatic microsomes to estimate in vitro inhibitory potency. The aims of this study were to compare \( K_i \) values determined in rat liver microsomes and freshly isolated rat hepatocytes of four cytochrome P450 (P450) inhibitors (clarithromycin, enoxacin, nelfinavir, and saquinavir) with known hepatic transporter involvement and a range of uptake (cell/medium concentration ratios 20–3000) and clearance (10–1200 \( \mu \)l/min/10\(^6\) cells) properties. Inhibition studies were performed using two well established P450 probe substrates (theophylline and midazolam). Comparison of unbound \( K_i \) values showed marked differences between the two in vitro systems for inhibition of metabolism. In two cases (clarithromycin and enoxacin, both low-clearance drugs), inhibitory potency in hepatocytes markedly exceeded that in microsomes (10- to 20-fold), and this result was consistent with their high cell/medium concentration ratios. For nelfinavir and saquinavir (high-clearance, extensively metabolized drugs), the opposite trend was seen in the \( K_i \) values: despite very high cell/medium concentration ratios, stronger inhibition was evident within microsomal preparations. Hence, the consequences of hepatic accumulation resulting from uptake transporters vary according to the clearance of the inhibitor. This study demonstrates that transporter-enzyme interplay can result in differences in inhibitory potency between microsomes and hepatocytes and hence drug-drug interaction predictions that are not always intuitive.

Introduction

Our understanding of the importance of hepatic transporters in controlling the disposition of drugs continues to evolve (Shitara et al., 2006; Niemi, 2007; Funk, 2008; Giacomini et al., 2010). The activity of members of the solute carrier and ATP-binding cassette families of transporter proteins may provide a mechanistic explanation for the limited success achieved for some drugs using standard (hepatic microsomal) in vitro experiments to predict pharmacokinetic aspects of clearance and drug-drug interaction (DDI) potential (Lam et al., 2006; Soars et al., 2007; Parker and Houston, 2008). Although various theoretical scenarios involving hepatic transporters can be proposed, specific examples with experimental confirmation of an unequivocal nature have been largely restricted to the statins (Lau et al., 2006; Shitara et al., 2006; Paine et al., 2008). It seems that the consequences of hepatic transporters may not always be intuitive because of transporter-enzyme interplay and “transporter-like” interactions involving other cellular processes.

The intact structural integrity of the hepatocyte may result in intracellular drug concentrations that differ from those in the surrounding medium but that are more representative of the in vivo situation. Assessment of the drug concentration available to the enzyme and/or transporter within the in vitro system is a key issue. Because accumulation of drugs in hepatocytes may occur via active uptake processes and/or intracellular binding, a concentration difference may exist between hepatocyte and hepatic microsomal incubations. Whether the drug is a bound or free entity within the cell is of importance; intracellular binding to sites not involved in the metabolic process may be of little consequence, as the free concentration within

ABBREVIATIONS: DDI, drug-drug interaction; P450, cytochrome P450; CLAR, clarithromycin; ENX, enoxacin; NFV, nelfinavir; SQV, saquinavir; YKpu, true hepatocyte/medium unbound drug concentration ratio reflecting purely distribution processes; \( K_{pu, \text{app}} \), apparent hepatocyte/medium unbound drug concentration ratio reflecting both elimination and distribution processes; 1,3-DMU, 1,3-dimethyluric acid.

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the cell will be in equilibrium with the external incubation media concentration (Grime and Riley, 2006; Poirier et al., 2008). However, in the case of uptake transporters, raising the cellular concentration in excess of the incubation medium concentration will result in more drug available to the enzyme. Resolution of this situation is of importance in both the assessment of clearance and the prediction of DDIs resulting from inhibition of drug clearance.

Comparison of $K_i$ data obtained in microsomes and hepatocytes could theoretically provide an indication of the mechanism of inhibitor accumulation. After appropriate binding corrections, more potent hepatocyte inhibition (as evident by lower $K_i$ values) would suggest the involvement of hepatic transporters because a higher concentration of unbound drug available for enzyme inhibition can be achieved compared with a similar incubation concentration in microsomes (Ito et al., 1998). Alternatively, similar values would indicate that any hepatic accumulation results from intracellular binding or lysosomal trapping (Grime and Riley, 2006; Hallifax and Houston, 2007). We recently demonstrated good concordance between $K_i$ values (range 0.05–30 μM) determined in rat hepatic microsomes and freshly isolated hepatocytes using seven P450 inhibitors (fluconazole, fluoxetine, fluvoxamine, ketoconazole, miconazole, omeprazole, and quinine) with a range of uptake properties (cell/medium concentration ratios 4–6000). These data suggest that the hepatic accumulation of these particular inhibitors results from intracellular binding rather than from the involvement of uptake transporters and indicate that microsomes and hepatocytes can be equivalent for determining their inhibitory potency (Brown et al., 2007). The source of these in vitro tissues was the rat to allow direct comparison of parameter values without the complication of intraindividual donor variability, which is often evident with human preparations (Hallifax et al., 2005; Rawden et al., 2005).

The aims of this study were to establish general scenarios for the impact of transporters on cellular concentrations via the use of comparative inhibition in isolated hepatocytes and hepatic microsomes. Four drugs were identified for study (CLAR, ENX, NFV, and SQV), all of which have known transporter involvement and show substantial hepatocellular accumulation (Sasabe et al., 1997; Yamano et al., 1999; Su et al., 2004; Maeda et al., 2007; Seithel et al., 2007; Parker and Houston, 2008; Giacomini et al., 2010). These drugs also show a range of metabolic clearance values (Table 1), allowing enzyme-transporter interplay to be assessed. The probes selected as markers of inhibition in the two systems were midazolam (for the CYP3A and transporter interplay to be assessed. The probes selected as markers of inhibition in the two systems were midazolam (for the CYP3A and

### Materials and Methods

**Chemicals.** SQV and NFV were generous gifts from Roche Products Limited (Welwyn, UK and Basel, Switzerland). 1′-Hydroxymidazolam and 4′-hydroxymidazolam were purchased from UFC Ltd. (Manchester, UK). All other chemicals and reagents used were of the highest grade available and were purchased from Sigma Chemicals (Poole, Dorset, UK) or BDH (Poole, Dorset, UK).

**Animal Source, Housing, and Diet.** Male Sprague-Dawley rats (240–260 g) were obtained from the Biological Sciences Unit, Medical School, University of Manchester (Manchester, UK). They were housed in groups of two to four, in opaque boxes on a bedding of sawdust in rooms maintained at a temperature of 20 ± 3°C, with a relative humidity of 40 to 70% and a 12-h light-dark cycle. The animals were allowed free access to CRM diet and fresh drinking water.

All animal protocols were approved by University of Manchester review committee.

**Microsomal Studies.** For hepatic microsomal preparations unanesthetized rats were sacrificed by cervical dislocation, and washed microsomes were prepared as described previously (Hayes et al., 1995). All kinetic and inhibition studies were performed in duplicate under initial rate conditions with respect to incubation time and microsomal protein concentration. All microsomal studies were performed using three independent batches of rat liver microsomes.

**Kinetic studies.** Theophylline (50–2500 μM) was preincubated with micromolar protein at 1 mg/ml and phosphate buffer (0.1 M, pH 7.4) for 5 min in Eppendorf tubes in a Thermomixer (Eppendorf AG, Hamburg, Germany) at 37°C and 900 rpm. Reactions were initiated by the addition of an NADPH-regenerating system and were terminated after an incubation time of 60 min by the addition of ice-cold acetonitrile containing an appropriate internal standard (S-mephyton). Experiments with midazolam were performed as described previously (Brown et al., 2007) at a protein concentration of 0.2 mg/ml over 10 min (appropriately defined linear conditions).

**Inhibition studies.** Either theophylline or midazolam was incubated at concentrations equivalent to 0.5 $K_{ii}$, $K_{ir}$ and $2K_{ir}$ with a range of inhibitor concentrations (at least 3 orders of magnitude to cover initial IC$_{50}$ estimates). Incubations were performed as described previously for the microsomal kinetic studies. Fluororesquonolone was shown to chelate divalent cations such as those present in an NADPH-regenerating system. For this reason, inhibition studies with ENX were performed in the presence and absence of regenerating system containing magnesium chloride, with no difference in inhibitory potency observed (data not shown).

**Hepatocyte Studies.** For hepatocyte preparations unanesthetized rats were sacrificed by cervical dislocation, and hepatocytes were prepared using an adaptation of the collagenase perfusion method as described previously (Hayes et al., 1995). Hepatocyte viability was determined using the trypan blue exclusion test, and only those hepatocyte preparations with viabilities greater than 85% were used. All kinetic and inhibition studies were performed in duplicate under initial rate conditions with respect to incubation time and hepatocyte density. All hepatocyte studies were performed using three independent hepatocyte preparations.

**Kinetic studies.** Theophylline (50–2500 μM) was preincubated with Williams’ medium E (pH 7.4) in Eppendorf tubes for 5 min in a Thermomixer set at 37°C and 900 rpm. Reactions were initiated by the addition of prewarmed hepatocytes (37°C) to give a final incubation concentration of 1 × 10⁶ cells/ml. Sixty minutes after incubation, reactions were terminated by snap-freezing in liquid nitrogen. Samples were then thawed, and ice-cold acetonitrile containing an appropriate internal standard (S-mephyton) was added. Experiments with midazolam were performed as described previously (Brown et al., 2007) at a cell concentration of 0.4 × 10⁶ cells/ml over 10 min (appropriately defined linear conditions).

**Inhibition studies.** Either theophylline or midazolam was incubated at concentrations equivalent to 0.5 $K_{ii}$, $K_{ir}$ and $2K_{ir}$ with a range of inhibitor concentrations (at least 3 orders of magnitude to cover initial IC$_{50}$ estimates). Incubations were performed as described previously for the hepatocyte kinetic studies.

**Determination of Metabolite Concentration.** Microsomal and hepatocyte samples were vortexed and centrifuged for 10 min at 11,600 g (Eppendorf

### TABLE 1

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>$K_i$ (μM)</th>
<th>$C_{i_{het}}$ hepatic microsomes</th>
<th>$f_u$ microsomes (at 1 μg/ml)</th>
<th>$f_u$ hepatocytes (at 10⁶ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enoxacin</td>
<td>20b</td>
<td>9.6</td>
<td>0.99c</td>
<td>0.99c</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>22c</td>
<td>16.9</td>
<td>0.81c</td>
<td>0.86c</td>
</tr>
<tr>
<td>Saquinavir</td>
<td>306d</td>
<td>4.86c</td>
<td>0.093d</td>
<td>0.14d</td>
</tr>
<tr>
<td>Nelfinavir</td>
<td>3352d</td>
<td>1290d</td>
<td>0.022d</td>
<td>0.035d</td>
</tr>
</tbody>
</table>

$C_{i_{het}}$, intrinsic clearance; $f_u$, fraction of unbound drug.

*Data from Yamano et al. (1999).

*J. Alder and J. D. Davis, unpublished data.

*Data from Parker and Houston (2008).
Under most experimental conditions any measurement of the tissue concentration of a drug represents total drug and reflects both intracellular binding and active uptake. Thus, a correction for intracellular binding needs to be made to estimate unbound cellular concentration and the impact of active uptake by transporters. One approach is to measure a cellular function that is dependent on the unbound drug concentration under disrupted conditions relative to normal conditions; for example, rate of transport at 4 and 37°C or rate of transport with and without an inhibitor.

When the tissue is an eliminating tissue, like the liver, there is the additional complication of metabolism and/or biliary efflux. Hence any measure of $K_{pu}$ may reflect not only the distribution processes but also additional terms for cellular clearance (Liu and Pang, 2005; Shitara et al., 2006; Webborn et al., 2007) and is an apparent term. For the liver the term $K_{pu,app}$ is appropriate:

$$K_{pu,app} = \frac{CL_{uptake} + CL_{passive}}{CL_{passive} + CL_{int1}}$$

where $CL_{int}$ is metabolic clearance.

To be fully descriptive, the denominator of eq. 2 should include a clearance term for efflux. However there is strong evidence for internalization of the apical membrane and down-regulation of these transporter proteins during the hepatocyte isolation procedure (Bow et al., 2008); hence it is not included in this model. For drugs for which active uptake is important, clearance due to passive diffusion will be a minor term in eq. 2. When clearance by metabolism is small, then little error will be involved; however, because the importance of clearance by metabolism increases (possibly exceeding the clearance for uptake), $K_{pu,app}$ has limited value as a distribution parameter.

An estimate of $K_{pu,app}$ for hepatocytes may be obtained by measuring a metabolic function. The ratio of the metabolic function in hepatocytes and microsomes would be appropriate (eq. 3). Given that the latter contains no cellular barrier, the ratio of the rates of metabolism reflect the $K_{pu,app}$:

$$K_{pu,app} = \frac{CL_{hepatocytes}}{CL_{microsomes}}$$

where $CL_{hepatocytes}$ and $CL_{microsomes}$ refer to the metabolic clearance in the respective in vitro system.

Likewise, the $K_{pu,app}$ may be determined from the ratio of $K_i$ data from microsomes and hepatocytes (eq. 4):

$$K_{pu,app} = \frac{K_i_{microsomes}}{K_i_{hepatocytes}}$$

After appropriate binding corrections, more potent hepatocyte inhibition (or clearance) would suggest the involvement of hepatic transporters because a higher concentration of unbound drug available for enzyme inhibition can be achieved compared with a similar incubation concentration in microsomes (Ito et al., 1998). Alternatively, similar values indicate that hepatic accumulation results from intracellular binding or lysosomal trapping.

### Results

**In Vitro Studies with Theophylline and Midazolam in Rat Freshly Isolated Hepatocytes in Suspension and Hepatic Microsomes.** For theophylline, the formation of 1,3-dimethyluric acid (1,3-DMU), the major metabolite in rat (McManus et al., 1988), is best described using biphasic kinetics in both hepatocytes and microsomes (Fig. 1), consistent with a high-affinity, low-capacity site and a low-affinity, high-capacity site (Table 2). When data are scaled to per...
gram per liver, hepatocyte clearance is approximately 6-fold higher than microsomal clearance. The $V_{\text{max}}$ is double and the $K_m$ is less than half that in hepatocytes when the high-affinity sites are compared. In addition, the dominance of the high-affinity site is more substantial in hepatocytes than in microsomes. The high-affinity $K_m$ values were used on the basis of selection of theophylline concentrations in subsequent inhibition studies.

For midazolam, substrate concentrations of 5, 15, and 30 µM were used in both microsomal and hepatocyte studies on the basis of previously established $K_m$ values (Brown et al., 2007). Both midazolam metabolites (1-OH-midazolam and 4-OH-midazolam) were monitored and control turnover rates are shown in Table 3. The predominant metabolite formed in both systems was found to be 4-OH-midazolam with approximately 3-fold more 4-hydroxylation than 1'-hydroxylation at each midazolam concentration studied, which is consistent with previous studies (Ghosal et al., 1996; Brown et al., 2007). There is no systematic difference between the control turnover rates in the two systems once the data are expressed per gram of liver (Table 3). Midazolam substrate concentrations for subsequent inhibition studies were selected on the basis of a $K_m$ value of approximately 10 µM.

### TABLE 3

<table>
<thead>
<tr>
<th>Midazolam conc.</th>
<th>Hepatocellular Rate of Formation</th>
<th>Microsomal Rate of Formation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1'-OH-midazolam</td>
<td>4-OH-midazolam</td>
</tr>
<tr>
<td>5 µM</td>
<td>5.3 ± 1.3</td>
<td>12.7 ± 4.2</td>
</tr>
<tr>
<td>15 µM</td>
<td>9.9 ± 1.5</td>
<td>25.4 ± 6.9</td>
</tr>
<tr>
<td>30 µM</td>
<td>11.2 ± 1.4</td>
<td>29.6 ± 7.9</td>
</tr>
</tbody>
</table>

### TABLE 4

$K_i$ values for inhibition of 1,3-dimethyluric acid formation from theophylline and inhibition of 1'- and 4-hydroxylation of midazolam in rat hepatocytes and hepatic microsomes

Mean data of three preparations ± S.D. are shown. Each preparation was studied with three substrate concentrations over a range of inhibitor concentrations.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Substrate</th>
<th>Pathway</th>
<th>Hepatocyte $K_i$</th>
<th>Microsomal $K_i$*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enoxacin</td>
<td>Theophylline</td>
<td>1,3-Dimethyluric acid</td>
<td>120 ± 65</td>
<td>2800 ± 1200</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>Midazolam</td>
<td>1'-Hydroxylation</td>
<td>75 ± 24</td>
<td>1000 ± 100</td>
</tr>
<tr>
<td>Saquinavir</td>
<td>Midazolam</td>
<td>4'-Hydroxylation</td>
<td>0.50 ± 0.035</td>
<td>0.22 ± 0.07</td>
</tr>
<tr>
<td>Nelfinavir</td>
<td>Midazolam</td>
<td>4'-Hydroxylation</td>
<td>0.47 ± 0.099</td>
<td>0.11 ± 0.01</td>
</tr>
</tbody>
</table>

* Statistical difference between hepatocytes and microsomes ($P < 0.01$).

**ENX Inhibition of 1,3-DMU Formation.** In liver microsomes, ENX displayed little significant inhibition of 1,3-DMU formation, even up to concentrations of 1000 µM. However, in hepatocytes, ENX inhibition is significantly greater than that in microsomes (Table 4). IC$_{50}$ plots for ENX in rat microsomes and hepatocytes are shown in Figs. 2, A and B, respectively, and exhibit a progressive increase in IC$_{50}$ value (84–256 µM) in hepatocytes as substrate concentration is increased, consistent with a competitive inhibition mechanism. Further analysis to obtain $K_i$ values confirmed that ENX displayed significantly more potent inhibition in hepatocytes (120 µM) than in microsomes (2800 µM), corresponding to a 24-fold difference.

**CLAR Inhibition of Midazolam Hydroxylation.** These characteristics are very similar to the inhibition of theophylline by ENX described above. CLAR showed weak inhibition of both pathways of midazolam metabolism in hepatic microsomes. 4'-Hydroxylation was more sensitive with 50% inhibition (in contrast to 30% inhibition of 1'-hydroxylation) at 1 mM CLAR. In contrast, hepatocyte inhibition was substantial, with both pathways showing evidence of competitive inhibition with IC$_{50}$ values increasing from 100 to 650 µM over the midazolam concentration range investigated. The $K_i$ values for hepatocytes were 60 and 75 µM in contrast to...
microsomal estimates of 650 and 1000 μM, for 4-OH- and 1’-OH-midazolam, respectively. Thus, the difference between in vitro systems amounted to >10-fold.

**NFV and SQV Inhibition of Midazolam Hydroxylation.** Typical IC₅₀ curves for the inhibition of the formation of 1’-OH-midazolam by NFV in hepatocytes and microsomes are shown in Figs. 3, A and B. Greater than 90% inhibition of midazolam hydroxylation was achieved at the highest concentration. IC₅₀ values for both inhibition of 4-OH- and 1’-OH-midazolam formation were found to progressively increase in a similar fashion (0.8–8.9 μM for hepatocytes and 0.7–2.9 μM for microsomes) with increasing midazolam concentration, indicating competitive inhibition. Ki values were calculated assuming a competitive inhibition model as 2.94 and 1.16 μM for the inhibition of 1’-OH-midazolam and 4-OH-midazolam formation, respectively, in hepatocytes. In microsomes, substantially lower Ki values (0.46 and 0.33 μM for the inhibition of the formation of 1’-OH-midazolam and 4-OH-midazolam, respectively) were found.

As with NFV, almost complete inhibition of midazolam hydroxylation by SQV was observed in both systems. SQV IC₅₀ values for 1’-OH-midazolam and 4-OH-midazolam formation both progressively increased with increasing midazolam concentration (1.38–3.45 μM for hepatocytes and 0.48–1.47 μM for microsomes), again indicating competitive inhibition. Ki values calculated using a competitive inhibition model were 0.50 and 0.47 μM for the formation of 1’-OH and 4-OH-midazolam, respectively, in hepatocytes and 0.22 and 0.11 μM for the formation of 1’-OH and 4-OH-midazolam, respectively, in microsomes.

**Comparison of Inhibition Profiles in Hepatocytes and Microsomal Systems.** The rank order of Ki values in rat hepatocytes and microsomes is essentially similar for the four inhibitors (Table 4). However marked differences in the ratio of Ki values between the systems are seen, ranging from 0.2 for NFV to 23 for ENX. Values of fraction unbound (fu) for each inhibitor in microsomes were determined experimentally (Table 1). These values were used to calculate the intracellular fraction unbound for the hepatocyte studies (Kilford et al., 2008). Both sets of values are listed in Table 1. Under the incubation conditions used, the ratios of the unbound fractions in the microsomal and hepatocyte incubations were within the range of 0.98 to 1.2; therefore, no corrections were made for the relative nonspecific binding in the two in vitro systems for any of the four inhibitors.

For each inhibitor, the ratio of the microsomal to hepatocellular Ki value was used as a estimate of Kpu,app. Table 5 compares these values with other independently determined Kp values, expressed both in terms of total and unbound drug concentrations (the latter obtained from permeability studies). With the exception of ENX, no concordance was evident, but rather an approximate negative correlation between Kpu,app and Kp based on total drug concentration. From the Kpu,app, two groupings are evident, depending on whether Kpu,app values are above or below unity. Both groups are also distinct in metabolic clearance, the first being low-clearance drugs (<3 ml/min/g liver), whereas the second are high-clearance drugs (>50 ml/min/g liver).

**Interplay of Metabolism and Transporters on Kpu.** The relationship describing Kpu,app in terms of uptake, passive permeability, and metabolism (eq. 2) was derived by others (Liu and Pang, 2005; Shitara et al., 2006) and was used to generate a three-dimensional surface for Kpu,app to illustrate the interplay of these clearance terms. Figure 4 shows the result for a range of uptake and metabolic clearances (0.001–10 ml/min/10⁶ cells) for drugs with intermediate (0.1 ml/min/10⁶ cells) passive permeability.

Taking the case in which clearances by uptake and metabolism are low and equal (0.001 ml/min/10⁶ cells) and less than passive permeability as a starting point, a Kpu,app of unity results. For compounds with a slow rate of metabolism, an increase in uptake clearance results in the expected progressive increase in Kpu,app from one to several thousand. By considering the same range of uptake clearance for compounds with faster metabolic clearances, Kpu,app values are lower but show a similar increase. At the extreme case of uptake clearance of 10 ml/min/10⁶ cells, the progressive decrease in Kpu,app as clearance via metabolism increases from a low value to unity. It is evident from this surface that only certain combinations of clearances

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**TABLE 5**

Comparison of hepatic and microsomal parameters for distribution, inhibition, and metabolism

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Kp</th>
<th>Kpu,app</th>
<th>Ki</th>
<th>1’-OH-midazolam</th>
<th>4-OH-midazolam</th>
<th>Theophylline Hydroxylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enoxacin</td>
<td>20</td>
<td>6.1</td>
<td>13</td>
<td>11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>22a</td>
<td>6.1</td>
<td>0.44</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saquinavir</td>
<td>306b</td>
<td>5.7</td>
<td>0.16</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nelfinavir</td>
<td>3352b</td>
<td>5.7</td>
<td>0.28</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

[a] Data from Yamano et al. (1999).
[b] Data from Parker and Houston (2008).
[c] Y. Yabe, unpublished data.
for uptake and metabolism result in $K_{pu, app}$ greater than 1. Because these two processes counteract each other, there are a number of situations in which $K_{pu, app}$ less than 1 results, although there is significant active uptake.

The slope of the surface is dependent on the permeability value assigned for the passive process. An intermediate permeability (0.1 ml/min/10^6 cells) is shown in Fig. 4: the use of a low permeability (0.001 ml/min/10^6 cells) results in a steeper surface and for a high permeability (10 ml/min/10^6 cells) the surface is almost flat.

It is useful to consider the surface (Fig. 4) in terms of two halves separated by the diagonal at $K_{pu, app}$ of unity for particular combinations of parameters. Above the diagonal, $K_{pu, app}$ is greater than 1, and compounds with clearance values within this half will be characterized by hepatic uptake that is metabolism rate-limited (e.g., ENX and CLAR). Compounds with properties corresponding to a position below the diagonal are those with transport rate-limited uptake (e.g., NFV and SQV). With the second scenario, for low permeability compounds with high metabolic clearance, there is no buildup of drug concentration within the cell; therefore, although transporters would be expected to result in high $K_p$ values; the rate-limiting step for drug disposition in the in vitro system is cellular uptake. However, when the latter process is substantially less than metabolic clearance, there is no cellular accumulation and $K_{pu, app}$ provides no guide to the importance of the transporter uptake. This contrasts with the first scenario for low-permeability compounds for which the impact of transporters is fully evident and $K_{pu, app}$ reflects $K_p$.

**Discussion**

For all four compounds, inhibition was clearly evident in the hepatocyte preparations, and this result is consistent with in vivo studies in both humans and rats (Edward et al., 1988; Olkkola et al., 1993; Davis et al., 1994; Yamaji et al., 1999; Yamano et al., 1999; Shibata et al., 2000). The two protease inhibitors studied, SQV and NFV, were very potent inhibitors of midazolam hydroxylation with $K_i$ values ≤1 μM. In the case of ENX and CLAR inhibition, $K_i$ values were much less potent in the region of 100 μM. All compounds are known to be substrates for the solute carrier transporter proteins (Sasabe et al., 1997; Yamano et al., 1999; Su et al., 2004; Maeda et al., 2007; Seithel et al., 2007; Giacomini et al., 2010), thus there was an expectation that hepatocytes would show higher sensitivity to inhibition than microsomes. Although this scenario was evident for ENX and CLAR, the opposite tendency was seen for the two protease inhibitors.

The marker substrates used in this study were theophylline and midazolam. They were selected as classic substrates for human P450s, namely CYP1A2 and CYP3A4. In the case of midazolam, this P450 preference is essentially maintained in the rat, in particular for the 4-hydroxylation pathway; however, in the case of the 1’-hydroxylation pathway there is also a contribution from CYP2C11 and CYP2C13 (Chovan et al., 2007). Theophylline appears to be a more promiscuous substrate in terms of rat cytochrome P450s (McManus et al., 1988); however, a high correlation has been reported between the in vivo clearance of theophylline and in vitro ethoxyresorufin O-deethylination (a classic CYP1A substrate reaction) (Matthew and Houston, 1990).

It has been suggested by Kobayashi et al. (2005) that there may be possible involvement of organic anion transporter inhibition in the interaction between theophylline and erythromycin. This suggestion was based on studies in Xenopus oocytes, demonstrating that both compounds have similar $K_i$ values and show competitive inhibition. Our data on theophylline metabolism in hepatocytes and microsomes do not confirm this proposal. Although clearance is higher in the cellular system, this is associated with substantial increases in $V_{max}$, a kinetic parameter not sensitive to substrate concentration changes. However, we cannot discount completely the possibility of a transporter component contributing to the theophylline ENX interaction.

ENX and CLAR inhibition in microsomes was very weak and only observable at very high concentrations. Our estimates of $K_i$, although somewhat imprecise, are certainly greater than 1000 μM. In contrast, with use of hepatocytes the inhibition properties of both compounds were easily characterized; classic IC50 profiles were obtained, and IC50 values showed a progressive increase with substrate concentration, indicating competitive inhibition. The $K_i$ values in hepatocytes for ENX and CLAR were approximately 100 μM, i.e., at least 10 times lower than those seen in microsomes.

The inhibitory properties of both ENX and CLAR in microsomes and hepatocytes contrast markedly with those observed for the protease inhibitors. In microsomes, SQV and NFV were shown to be potent inhibitors of the CYP3A-mediated metabolism of midazolam. The relative inhibitory potency was very similar for both 1’-OH-midazolam and 4-OH-midazolam formation; the formation of 4-OH-midazolam was found to be more sensitive to inhibition than that of 1’-OH-midazolam. SQV and NFV exhibited competitive inhibition and no evidence of partial inhibition. The inhibitory potency for the inhibition of 1’-OH-midazolam observed in rat microsomes is comparable to results published by Shibata et al. (2000).

The inhibition potency of NFV and SQV observed in rat hepatocytes was less than that evident with microsomes for both metabolites; $K_i$ values ranged from 0.47 to 2.94 and 0.11 to 0.46 μM for the hydroxylation of midazolam in hepatocytes and microsomes, respectively. This was particularly apparent in the case of NFV and the trend is reflected in SQV $K_i$ values.

Both ENX and CLAR show low clearance by the liver, and in the case of ENX renal clearance is equally important in the overall elimination of this compound in vivo in both rats and man (Paton and Reeves, 1988; Davis et al., 1994). In contrast, the hepatic clearance of the protease inhibitors is very high (Shibata et al., 2000; Parker and Houston, 2008). We have studied this in some detail in vitro and have
demonstrated that in hepatocytes the metabolic clearance is transporter rate-limited (Parker and Houston, 2008). Thus, there is a clear distinction between these two classes of compound in terms of their hepatic metabolic clearance characteristics. This behavior is consistent with the differences reported here in terms of $K_i$ values and the resulting $K_p_{ape}$ values. Interestingly, the ratio of microsomal to hepatocyte $K_m$ values (Parker and Houston, 2008) is similar to the corresponding ratio of $K_i$ values (Table 5).

The data reported herewith contrast with our previous publication (Brown et al., 2007), in which we were able to show that the hepatic accumulation of seven P450 inhibitors (fluconazole, fluoxetine, fluvoxamine, ketoconazole, miconazole, omeprazole, and quinine) resulted from intracellular binding rather than from any involvement of transporters. For these compounds we demonstrated good concordance between $K_i$ values (range 0.05–30 μM) in microsomes and hepatocytes despite the differing importance of hepatic accumulation observed for these compounds ($K_p$ values ranging from 4 to 6000). In contrast, Grime et al. (2008) demonstrated a 5-fold difference in inhibition between recombinant rat P450s and isolated rat hepatocytes for four lipophilic carboxylic acids including atorvastatin and pitavastatin. As with the present study there was a disconnect between the change in the $K_i$ value between these systems and the extent of hepatic accumulation (approximately 1000-fold). Thus, the authors concluded that there was high intracellular binding, resulting in accumulation and that proposed $K_p$ values would be on the order of 5, a situation similar to that observed with NFV and SQV (Parker and Houston, 2008).

The IC50 curves reported by Grime et al. (2008) were somewhat flat, contrasting with the traditional sigmoidal shape, and the reasons for this are open to question. These authors discussed the scenario in which the affinity constant for the transporter may be of a value similar to that observed with NFV and SQV (Parker and Houston, 2008). The $K_m$ values reported here are as expected on the basis of information available on the active transport of these compounds into hepatocytes. In contrast, the observations with the pro tease inhibitors were not intuitive; however, consideration of hepatic clearance allows these observations to be rationalized. Use of a simple model to describe the hepatocellular processes of metabolism, uptake, and passive permeability illustrates the interplay that defines $K_p_{ape}$. In addition, it provides a rationale for two inhibition scenarios resulting from whether the inhibitor displays a metabolic or uptake rate-limited clearance and highlights the potential shortcomings of using subcellular fractions such as microsomes rather than hepatocytes to predict likely DDI in vivo.

Acknowledgments. We thank Sue Murby and Dr. Raj Badhan for valuable assistance with the liquid chromatography-tandem mass spectrometry and simulation work.

References


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