In Vitro and in Silico Identification and Characterization of Thiabendazole as a Mechanism-Based Inhibitor of CYP1A2 and Simulation of Possible Pharmacokinetic Drug-Drug Interactions

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ABSTRACT:

Thiabendazole (TBZ) and its major metabolite 5-hydroxythiabendazole (5OH-TBZ) were screened for potential time-dependent inhibition (TDI) against CYP1A2. Screen assays were carried out in the absence and presence of NADPH. TDI was observed with both compounds, with k inact and K I values of 0.08 and 0.02 min⁻¹ and 1.4 and 63.3 μM for TBZ and 5OH-TBZ, respectively. Enzyme inactivation was time-, concentration-, and NADPH-dependent. Inactivation by TBZ was irreversible by dialysis and oxidation by potassium ferricyanide, and there was no protection by glutathione. 5OH-TBZ was a weak TDI of CYP1A2, and enzyme activity was recovered by dialysis. IC₅₀ determination of TBZ and 5OH-TBZ showed both compounds to be potent inhibitors, with IC₅₀ values of 0.83 and 13.05 μM, respectively. IC₅₀ shift studies also demonstrated that TBZ was a TDI of CYP1A2. In silico methods identified the thiazole group as a TDI fragment and predicted it as the site of metabolism. The observation pointed to epoxidation of the thiazole and the benzyl rings of TBZ as possible routes of metabolism and mechanisms of TDI. Drug-drug interaction (DDI) simulation studies using SimCyp showed good predictions for competitive inhibition. However, predictions for mechanism-based inhibition (MBI)-based DDI were not in agreement with clinical observations. There was no TBZ accumulation upon chronic administration of the drug. The in vitro MBI findings might therefore not be capturing the in vivo situation in which the proposed bioactivation route is minor. This might be the case for TBZ in which, in vivo, UDP glucuronosyltransferases and sulfotransferase metabolize and eliminate the 5OH-TBZ.

Thiabendazole (TBZ) is a broad-spectrum anthelmintic for many animal species and is used to treat parasitic infections in humans (Brown et al., 1961; Hennekeuser et al., 1969; Walton et al., 1999). It has also been used as an agricultural fungicide for pre- and postharvest treatment of fruit and vegetables and as a preservative in many consumer food products (Szego et al., 1993; Arenas and Johnson, 1994; Walton et al., 1999; Groten et al., 2000). Studies have shown that thiabendazole is extensively metabolized in humans and in animals (Tocco et al., 1966). Several routes of biotransformation of thiabendazole have been proposed (Fig. 1). The major route is the CYP1A2-catalyzed hydroxylation to 5-hydroxythiabendazole, which is further metabolized to glucuronide and sulfate conjugates (Coulet et al., 1998). Other metabolites include 4-hydroxythiabendazole, 2-acetylbenzimidazole, N-methylthiabendazole, and benzimidazole (Fujitani et al., 1991). TBZ has also been shown to induce members of the CYP1A and CYP2B family in rats in vivo (Price et al., 2004) and rabbit CYP1A2 in vitro (Aix et al., 1994), which implies the possibility of the compound inducing its own metabolism. However, TBZ has not been shown to induce CYP1A2 in humans in vitro (Bapiro et al., 2002). No studies have been done on its potential to induce CYP1A2 in vivo in humans.

Although TBZ is considered a safe drug in humans, studies in mice have shown some toxicity. It has been associated with nephrotoxicity, resulting in severe kidney damage (Mizutani et al., 1990; Tada et al., 1992; Fujitani et al., 1999); and teratogenicity, resulting in impairment of limb development and toxicity to the embryo (Ogata et al., 1984). Isolated cases of hepatotoxicity have also been reported in humans after TBZ administration (Manivel et al., 1987; Bion et al., 1995). Toxicity to the embryo has been associated with covalent binding of

ABBREVIATIONS: TBZ, thiabendazole; P450, cytochrome P450; CHC, 3-cyano-7-hydroxycoumarin; TDI, time-dependent inhibition/inhibitor; MBI, mechanism-based inhibition/inhibitor; CEC, 3-cyano-7-ethoxycoumarin; fm, fraction metabolized; 5OH-TBZ, 5-hydroxythiabendazole; AUC, area under the curve; ACN, acetonitrile.
reactive metabolite from cytochrome P450 (P450)-mediated metabolism to tissue protein in the embryo (Yoneyama and Ichikawa, 1986). TBZ-induced nephrotoxicity is believed to be caused by thioformamide formed from oxidative cleavage of thiazole moiety in thiabendazole by P450. Covalent binding of metabolites to cellular macromolecules has been associated with drug toxicity, and thiazole cleavage has been associated with toxicity via bioactivation by P450 to form epoxides (Mizutani et al., 1994). Its possible role and mechanisms of teratogenicity and hepatotoxicity in humans are still not clear.

Many of the toxic effects of TBZ have been linked to reactive metabolites, and several mechanisms of bioactivation of the major metabolite 5-hydroxythiabendazole have been proposed (Mizutani et al., 1993; Coulet et al., 2000). In rats, TBZ has also been shown to cause depletion of renal and hepatic glutathione in vivo (Mizutani et al., 1990), suggesting the production of reactive metabolites during thiabendazole metabolism. In one study, it was shown that 5-hydroxythiabendazole undergoes oxidation by P450 to form electrophilic species that can be trapped by glutathione (Dalvie et al., 2006).

A recent study has shown the potential for the inhibition of drug metabolism by TBZ to cause clinically important interactions with CYP1A2 substrates (Bapiro et al., 2005). TBZ has been shown to increase theophylline serum levels (2–3-fold, resulting in serious side effects) (Lew et al., 1989; Schneider et al., 1990). It has also been shown to be a potent inhibitor of CYP1A2 in vitro, with a $K_i$ value of 1.54 μM (Bapiro et al., 2001). Using an experimental setup that evaluates reversible inhibition, the mode of inhibition was thought to be of mixed mechanisms.

Many of the observations in the previous studies, for example, covalent binding to protein, irreversible binding to tissue protein both in vivo and in vitro in a time- and concentration-dependent manner, and further metabolism of the main metabolite to reactive metabolites give a strong suggestion that the compound could be a likely TDI. TDI is a type of enzyme inhibition characterized by time- and concentration-dependent loss of enzyme activity (Silverman, 1995). Mechanism-based inhibition (MBI) is a type of TDI in which inhibition is permanent and restoration of activity is only by synthesis of new enzyme. MBI inactivates the P450 enzymes by either binding covalently to the heme or binding to apoprotein and by heme chelation. Reactive metabolites have been implicated in TDI and have been associated with clinically important drug-drug interactions and toxicity via immunogenic drug-protein conjugates. In this present study, the aim was to determine whether thiabendazole is a TDI using in vitro methods and to identify likely sites of metabolism implicated in TDI in silico. Potential drug-drug interactions via this mechanism were also simulated.
assays on the effect of TBZ and 5OH-TBZ on CYP1A2 and TBZ on CYP2C9 and 3A4 activities (Table 1).

**IC50 determination.** The IC50 is the inhibitor concentration that is required to reduce enzyme activity by half. The assay was conducted in a similar way to the time-dependent screen assay. Varying concentrations of thiabendazole (0.02, 0.1, 0.2, 0.5, 1.5, 4.4, 13.3, and 40 μM) were preincubated both in the presence and absence of NADPH. The activity assay was then performed, and activity was measured. The experiments were done with a high substrate concentration (CEC) of 12 μM to minimize the contribution of competitive inhibition. For the competitive IC50 assay, there was no preincubation step. Varying concentrations of TBZ and 5OH-TBZ (40, 20, 10, 5, 2.5, 1.25, 0.625, and 0.31 μM) were incubated with enzyme (0.5 pmol/well), KPO4, pH 7.4 (0.1 M), and substrate at Km concentration (3 μM). The reaction was initiated by the addition NADPH (1 mM) and terminated by an ice-cold 20% Tris base/80% ACN solution after 15 min.

**Kinetics of CYP1A2 inactivation by thiabendazole.** The two-step incubation method was used to characterize the time- and concentration-dependent inhibition of CYP1A2 by TBZ and 5OH-TBZ. In the inactivation assay, varying concentrations of TBZ (ranging between 0 and 20 μM) and SOH-TBZ (ranging between 1.56 and 200 μM) were incubated with CYP1A2 (25 pmol/ml), NADPH, and 0.1 M phosphate buffer, pH 7.4. At selected preincubation times, an aliquot of the preincubation mix (1:10 dilution) was taken and added to the activity assay (similar to the assay in the TDI screen assay), and a further 15-min incubation was done at 37°C. The reaction was terminated by addition of ice-cold 20% Tris base/80% ACN solution, and activity was followed by measuring formation of CHC.

**Effects of glutathione on inactivation of CYP1A2 by thiabendazole.** The inactivation of CYP1A2 by TBZ was investigated in the presence of glutathione (5 mM), an electrophile-trapping agent. The glutathione was added together with TBZ (1 and 20 μM) to the inactivation assay both in the presence and absence of NADPH. The activity assay was then performed, and the activity of enzyme followed by measuring fluorescence of CHC. Control activities were determined in the absence of TBZ.

**Effect of potassium ferricyanide on inactivation of CYP1A2.** The assay was performed to determine whether the catalytic function of recombinant CYP1A2 could be restored after oxidation by potassium ferricyanide (2 mM in 0.1 M phosphate buffer, pH 7.4). The combined effect of potassium ferricyanide and glutathione was also investigated, and in this case the glutathione was added in the inactivation assay. The experiment was divided into three parts: the inactivation assay (preincubation), restoration of activity assay, and the activity assay. An aliquot (50 μl) was taken from the inactivation assay after 10 min and added to the restoration of activity plate, which had 50 μl of the potassium ferricyanide (0.1 M phosphate buffer, pH 7.4, for the controls). After a further 10-min incubation, another 10-μl aliquot was taken and added to the activity assay plate consisting of fresh 0.1 M phosphate buffer, pH 7.4, 1 mM NADPH, and 12 μM CEC. The reaction was then terminated by addition of ice-cold 20% Tris base/80% ACN solution, and activity followed by measuring the formation of 3-cyano-7-hydroxycoumarin (CHC).

**Effect of dialysis.** The two-step assay was adapted to determine the effect of dialysis. In the inactivation assay, 25 pmol/ml CYP1A2 was incubated with NADPH and either 1 μM furafylline, 20 μM fluvoxamine, 20 μM TBZ, 20 μM 5OH-TBZ, or 0.2% dimethyl sulfoxide (vehicle control) in 0.1 M phosphate buffer, pH 7.4. The incubation mixtures were transferred to Slide-A-Lyzer minidialysis units with a molecular weight cut-off of 10,000 (Pierce Chemical, Rockford, IL). Dialysis was performed at 4°C for 4 h in 1 l of 50 mM potassium phosphate buffer, pH 7.4. The dialysis buffer was changed every hour. Parallel analysis was done with incubation mixes that were stored at 4°C for the duration of the dialysis experiment. Samples were then analyzed for activity as described above.

### Data Analysis

**Determination of the normalized ratio.** In the screen assay, the effect of each inhibitor on the activity of the enzyme were expressed as the normalized ratio calculated as shown in the equation below. The classification was based on the method by Atkins et al. (2005):

\[
\text{Normalized ratio} = \frac{(R + 1 \text{NADPH})/(R - 1 \text{NADPH})}{(R + 1 \text{NADPH})/(R - 1 \text{NADPH})}
\]

where \(R + 1 \text{NADPH}\) is the rate of reaction when incubation is performed in the presence of both inhibitor and NADPH, \(R - 1 \text{NADPH}\) is the rate of reaction when incubation is performed in the presence of NADPH but in the absence of the inhibitor, \(R + 1 \text{NADPH}\) is the rate of reaction when incubation is performed in the presence of inhibitor but in the absence of NADPH, and \(R - 1 \text{NADPH}\) is the rate of reaction when incubation is performed in the absence of both inhibitor and NADPH. Compounds with a normalized ratio below 0.7 were classified as TDI, those with normalized ratio above 0.9 as non-TDI, and those with a normalized ratio lying between 0.7 and 0.9 fell in the gray zone in which their status cannot be clearly defined.

**Determination of Kf and kfmax.** The natural logarithm of the percentage of remaining activity was plotted against the preincubation time at each inhibitor concentration to obtain the kobs (slope). The kobs is the rate constant describing the inactivation at each inhibitor concentration. Nonlinear regression (GraphPad Prism; GraphPad Software Inc.) was then used to estimate the kfmax and the Kf from the equation below:

\[
k_{\text{obs}} = \frac{k_{\text{fmax}} \times [I]}{K + [I]}
\]

where \(k_{\text{fmax}}\) is the maximal rate of inactivation, \(K_f\) is the inhibitor concentration required for half-maximal inactivation, and [I] is the preincubation concentration of inhibitor.

### In Silico Experiments

**Substructure search and site of metabolism prediction.** A TDI substructure search was done before site of metabolism prediction to identify chemical groups in TBZ that are likely to cause TDI. An in-house script (S. Winiwarter, unpublished data) was used to identify substructures associated with TDI on thiabendazole. TBZ was then submitted to MetaSite version 2.7.5 to predict the site(s) of metabolism. MetaSite is a fully automated program that considers structural complementarity between the enzyme active site and the ligand and comes up with the most optimal orientation. Both the protein active site and the ligand are represented by selected distance-based descriptors using molecular interaction fields computed in GRID. The site of metabolism is described by a probability index that is a product of similarity between ligand and protein. In this study, default parameters were used, and top three averaged rankings with the reactivity component enabled were considered.

**Docking Studies.** GLUE. Thiabendazole was docked into the active site of CYP1A2 crystal structure (PDB 2HI4), with the crystallographic water molecules. The docking experiment and analysis of the ligand-receptor interaction were performed by GLUE, a GRID-based docking program. The program

<table>
<thead>
<tr>
<th>Substrate Conc.*</th>
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<th>Negative Control</th>
<th>Excitation Wavelength</th>
<th>Emission Wavelength</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A2 25 pmol/ml</td>
<td>Furfuryline</td>
<td>Fluvoxamine</td>
<td>405</td>
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</tr>
<tr>
<td>2C9 300 pmol/ml</td>
<td>MFC</td>
<td>Sulfaphenazole</td>
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<td>535</td>
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<tr>
<td>3A4 50 pmol/ml</td>
<td>BFC</td>
<td>Troleandomycin</td>
<td>405</td>
<td>535</td>
</tr>
</tbody>
</table>

* BFC, 7-benzyloxy-4-trifluoromethylcoumarin; MFC, 7-methoxy-4-trifluoromethylcoumarin.

**IC50** determination.

**Substrate concentration at 4x the K<sub>m</sub> value (to reduce the effect of reversible inhibition).**
maps the active site using hydrophobic, hydrogen bond donor/acceptor, and electrostatic probes. Before docking, the ligand (ANF) was removed from the active site of the crystal structure. A dummy molecule, incapable of accepting hydrogen bonds was added above the heme iron to inactivate interactions between the docked compound and the heme. The PDB file was modified in GREATER, which converted the PDB file format to input files required for the docking procedure (kout files). Default parameters were used for the docking procedure.

GOLD. Before docking, thiabendazole was built and energy minimized in vacuo using MMF94s force field and MMF94s charges using the conjugated gradient method. The active site was defined with a radius of 15 Å from the heme iron of the protein and for all other parameters default settings were used. The genetic algorithm implemented in GOLD was then used to optimize orientation of the ligand into the active site. During the optimization, the ligand was considered flexible and the enzyme active site was rigid. Ten dockings were allowed with an early termination if the root mean square distances were within 1.5 Å for the top three solutions.

**In Silico DDIs: Pharmacokinetic Simulations.** SimCyp population based ADME simulator version 8.1 was used to simulate the in vivo effects of thiabendazole on elimination of itself and other CYP1A2 substrates: caffeine and theophylline. In general, the simulation process involved uploading of the pharmacokinetic and enzyme kinetic data for thiabendazole. The pharmacokinetics data of the CYP1A2 substrate drugs were already uploaded in the SimCyp software. In vivo coadministration of thiabendazole with CYP1A2 substrate drugs was only available for caffeine and theophylline. This was followed by modeling where we mimicked published experimental methodology (e.g., dose, interval, duration, sample size) when the thiabendazole was administered alone (Tocco et al., 1966) or in combination with theophylline (Schneider et al., 1990) and caffeine (Bapiro et al., 2005). A fasted virtual Caucasian population was used in all the simulations. Clinical trial sample sizes of 10 were used in all evaluations. The oral route of drug administration was considered in this scenario for all the DDIs tested, and an additional theophylline-thiabendazole interaction was also simulated after theophylline infusion (Schneider et al., 1990).

**Competitive inhibition.** In these simulations, thiabendazole was the competitive inhibitor, with a $K_I$ value of 1.54 µM as determined in our previous studies (Bapiro et al., 2001). The affected drugs were caffeine and theophylline given at doses used clinically. A single oral dose pharmacokinetic trial design was considered in all simulations that involved competitive CYP1A2 inhibition.
MBI. Clinical drug doses, frequency and duration of 3 days (thiabendazole is dosed twice daily for 3 days), were uploaded at trial design stage. The effect of thiabendazole on its own elimination was also tested after 3 days of administration. In addition, thiabendazole-theophylline interactions were simulated following published data (Schneider et al., 1990).

Results

TDI Screen Assay. The effect of TBZ on the activities of CYP1A2, CYP2C9, and CYP3A4 and 5OH-TBZ on CYP1A2 were investigated. As shown in Figs. 2 and 3, thiabendazole was a clear TDI for CYP1A2 but not for CYP2C9, and it was unclear for CYP3A4. 5OH-TBZ was a weak TDI for CYP1A2 as indicated by the normalized ratio. There was no concentration dependence on the effect of TBZ on CYP3A4, making the compound unlikely to be a TDI of this enzyme. As expected, the negative controls (reversible inhibitors) were clearly non-TDI and the positive controls (known TDI) used for each isoform were picked up by the assay showing clear TDI. No significant loss of activity was observed in the control experiments in which there was no inhibitor.

IC50 Determination. Inhibition of CYP1A2 by TBZ and 5OH-TBZ was evaluated using two assays: the activity assay and the two-step TDI assay. The activation assay was an indication of the effects of TBZ and 5OH-TBZ via competitive inhibition. Both compounds were potent inhibitors of CYP1A2, with IC50 value of 0.83 and 13.05 μM for TBZ and 5OH-TBZ, respectively, as shown in Fig. 4. The two-step assay was done for TBZ. The assay was based on the fact that TDI causes a decrease in the IC50 value when preincubated with NADPH. Because TBZ had been shown to be a potent TDI in the TDI assay described above, the assay was used to further confirm the observed result. TBZ inhibited CYP1A2 activity, with an IC50 value of 84.5 μM when preincubation was done in the absence of NADPH. Preincubation with NADPH increased the inhibition of CYP1A2 considerably (Fig. 5), giving a further strong indication that TBZ is a time-dependent inactivator of CYP1A2. The IC50 value was lowered to 2.8 μM.

Kinetics of CYP1A2 Inactivation by TBZ and 5OH-TBZ. Kinetics of CYP1A2 inactivation by TBZ and 5OH-TBZ was followed by measuring loss of CEC dealkylation activity (Fig. 6, a and b). Inactivation of CYP1A2 was in a time- and concentration-dependent manner and followed pseudo-first order kinetics. The time course for the inactivation is shown in Fig. 6a. Nonlinear regression analysis of the time course data was then used to determine the initial rate constants for the inactivation at various concentrations of the two compounds (Fig. 6b). Inactivation constants k_inact and K_I for CYP1A2 were determined to be 0.08 min⁻¹ and 1.4 μM, respectively, for TBZ and 0.02 min⁻¹ and 63.03 μM for 5OH-TBZ, respectively. At higher concentrations of thiabendazole, some loss of activity was observed at the zero point because of the carryover of thiabendazole into the inactivation assay mixture. Because 5OH-TBZ is a weak inhibitor, there were no significant changes in the activity of the enzyme at the varying time points for the lower concentrations (1.6, 3.1, and 6.3 μM). However, the changes were much clearer at the higher concentrations, giving clearer slopes (Fig. 6a).

Effect of Glutathione and Potassium Ferricyanide on the Inactivation of CYP1A2. Protection of the enzyme inactivation by glutathione and restoration of activity by oxidation by potassium ferricyanide was investigated both in the presence and absence of NADPH.
There was no significant increase in activity in the presence of glutathione, an indication that glutathione failed to protect the enzyme from inactivation. Potassium ferricyanide was not able to restore enzyme activity, giving an indication that the inhibitor was not displaced from the enzyme. Inactivation was not significant when NADPH was absent in the preincubation step. There was no significant change when the compounds were used in combination.

**Effect of Dialysis.** To determine whether the inactivation effects by TBZ and 5OH-TBZ were reversible, compounds were incubated with CYP1A2 as described under Materials and Methods. For comparison, the experiment was also conducted with samples that were not dialyzed. As indicated in Fig. 8, the dialysis did not affect the magnitude of inactivation by furafylline, which is a known MBI of CYP1A2. The effects of fluvoxamine were greatly reduced by dialysis, and activity was restored to 100%. It was clear that thiabendazole was an irreversible inhibitor, because there was no significant restoration of activity after dialysis. SOH-TBZ was a clear reversible inhibitor.

**Substructure Search and Site of Metabolism Prediction.** Based on substructures associated with TDI (Fontana et al., 2005), two substructures were identified: a conjugated system and the thiazole ring (Fig. 9a). These substructures have been associated with mechanism-based inhibition, where they are metabolized to reactive metabolites that bind irreversibly to the enzyme (Fontana et al., 2005). The compound was then submitted to MetaSite, which is a program that predicts the likely site of metabolism. The program has been proven to be able to predict the likely site metabolism within the top three predictions in 80% of the cases in structurally diverse compounds (Cruciani et al., 2005). MetaSite predicted the 5C on the benzyl group of thiabendazole as the top ranked site of metabolism (Fig. 9b). This is in agreement with what has been experimentally determined. It has been shown that the main route of metabolism of thiabendazole in both humans and animals is hydroxylation to 5-hydroxythiabendazole, and CYP1A2 is the main enzyme involved (Coulet et al., 1998). The thiazole ring that has been associated with the TDI effects in other compounds, e.g., ritonavir (Fontana et al., 2005), was also predicted as the site of metabolism in the top three rankings. Studies have shown that the thiazole ring as found in thiabendazole can be cleaved to form epoxide intermediates and thioamides (Mizutani et al., 1994), with the former metabolites being implicated in TDI (Fontana et al., 2005).

**Docking Studies.** The results obtained from the docking experiments were in agreement with the site of metabolism predictions. There was one preferred orientation in all the three GOLD solutions in which the benzene ring of the compound was closest to the heme catalytic center at an average distance of 4.5 Å in all the three solutions. One of the three docking poses is shown (Fig. 10).
GLUE, eight of the 10 docking solutions were in agreement with the site of metabolism prediction. In five of the 10 solutions, the thiazole ring was toward the heme and in three the benzene was the one closest to the heme catalytic center. In two of the solutions, both predicted groups were too far away from the catalytic center for any metabolism to occur. Examples of the possible docking poses are shown (Fig. 11). Interactions of the benzene ring or the thiazole ring of thiabendazole with phenylalanine 226 of CYP1A2 seem to be important in determining the orientation of thiabendazole in the enzyme active site.

**TABLE 2**

<table>
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<tr>
<th>Combination</th>
<th>Mechanism</th>
<th>Predicted Mean AUC - Fold Increase</th>
<th>Observed Clinical DDI - Fold Increase in AUC</th>
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<tr>
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**FIG. 11.** Examples of different orientations in which thiabendazole docks into the active site of CYP1A2. The docking experiment was performed in GLUE as described under Materials and Methods. The color coding is the same as described for legend 7. In five of the top 10 ranked solutions, the thiazole group was the group closest to the heme (b); in three solutions, the benzene ring in which hydroxylation occurs was closest (a); and in two solutions, both groups were further away (c). Interactions of the benzene or thiazole moiety of thiabendazole with phenylalanine 226 of CYP1A2 seems to be important in determining the orientation of thiabendazole in the enzyme active site.

**DDI Pharmacokinetic Simulations.** Table 2 shows the simulated DDI interactions involving thiabendazole as a competitive and as an irreversible inhibitor of CYP1A2. The MBI effects were significantly more profound compared with the competitive effects for the CYP1A2 substrate tested.

**Discussion**

TBZ had been shown to be a strong inhibitor of CYP1A2 activity in vitro and in vivo. It has also been suggested that its major metabolite 5OH-TBZ can be bioactivated to reactive metabolites that might be associated with toxic effects. In this study, TBZ was screened for potential TDI against CYP2C9 and CYP3A4 only based on a significant number of compounds having shown TDI against these enzymes. The choice of CYP1A2 was based TBZ having been demonstrated to be a substrate and a mixed type inhibitor of the enzyme.

The screen assay managed to differentiate between the known-TDI and non-TDI compounds, validating results obtained for TBZ and 5OH-TBZ (Fig. 3). TBZ and 5OH-TBZ were clear TDI for CYP1A2, with TBZ being a stronger TDI than 5OH-TBZ. The inactivation of CYP1A2 by TBZ and 5OH-TBZ was concentration-, time-, and NADPH-dependent (Fig. 6), an indication the inactivation occurred via a catalytic process. The $K_I$ value determined for TBZ (1.4 $\mu$M) was lower than the total plasma concentrations the drug is capable of reaching (19 $\mu$M) (Bapiro et al., 2005). The $k_{inact}$ value of 0.08 $min^{-1}$ is relatively high, such that the resulting $k_{inact}/K_I$ of 0.05 $\mu$M/min is predictive of likely significant enzyme inactivation. In the treatment of strongyloidiasis in humans, the drug is given over 3 days at 25 mg/kg/day (Merck, 1999; Satoh and Kokaze, 2004), thus providing the time component during which CYP1A2 will progressively be
inactivated. However, in a chronic exposure study to thiabendazole (50 mg/kg/day), the pharmacokinetic studies did not show accumulation of thiabendazole (Bauer et al., 1982).

TBZ showed much lower potency when preincubated in the absence (IC_{50} = 84.5 μM) compared with the presence of NADPH (IC_{50} = 2.8 μM) (Fig. 5). This shift in the IC_{50} value further suggests that TBZ is a likely TDI. However, it should be noted that the high value (84.5 μM) cannot be compared with the low IC_{50} values (1.2 and 0.83 μM) obtained in the reversible inhibition assay (Bapiro et al., 2001), because the inhibitor is diluted 10-fold before the inhibition is allowed to occur. A high substrate concentration was used to minimize competitive inhibition, hence the high IC_{50} value in our study. There was no protection of CYP1A2 from inactivation by TBZ in the presence of the nucleophilic trapping agent glutathione, an indication that reactive intermediates were not escaping the active site before inactivation. This is one of the characteristics that differentiate irreversible inhibitors from the reversible inhibitors. In irreversible inhibitors, glutathione will have no effect in preventing inactivation because reactive species formed during the enzymatic reaction react rapidly with amino acids in the active site rather than diffuse out.

Dialysis experiments were done to further confirm the mechanism of CYP1A2 TDI by TBZ and 5-OH TBZ. Approximately 20% of CYP1A2 activity was restored after dialysis after inactivation by TBZ (Fig. 8), suggesting the mixed mode of inhibition. There was no restoration of activity when furafylline, a positive control MBI, was used to inhibit CYP1A2. Activity was restored for the reversible inhibitor flavoxamine. Significant recovery was demonstrated for 5OH-TBZ, suggesting the generation of reversible inhibitory metabolite(s) in vitro. The results also confirm that 5OH-TBZ is a weak TDI and TBZ a true MBI. This has important implications for the proposed bioactivation path (Fig. 1) in which the route of TDI-associated metabolism might not go through 5-OH TBZ but directly from TBZ, as proposed in Fig. 12.

In silico results were in agreement with each other and with observations from literature. The first ranked site of metabolism was on the 5C of the benzene ring, a site where metabolism has been shown to take place (Coulet et al., 1998). All three docking poses in GOLD and three of 10 docking poses in GLUE showed the site at a favorable distance to the heme catalytic center. The thiazole group was predicted as the likely site of metabolism by both MetaSite, and five of 10 docking poses in GLUE had the group close the heme. In the other two docking poses, both predicted sites of metabolism were too far away from the catalytic center.

TBZ has been reported to be a potent and mixed inhibitor of CYP1A2 both in vitro and in vivo (Bapiro et al., 2001, 2005). The docking results could explain the observed results. When the compound is docked with the benzyl moiety oriented toward the heme (Fig. 11a), hydroxylation to 5-hydroxythiabendazole, which is the main route of metabolism, is favored. The docking solution could explain the competitive inhibitory effects of thiabendazole on CYP1A2. When the thiazole moiety docks close to the heme (Fig. 11b), bioactivation of thiabendazole could likely result in TDI. When TBZ docks in the active site cavity but far away from the reactive center (Fig. 11c), noncompetitive inhibition could result from allosteric binding. Therefore, it can be assumed that depending on the conditions in vivo and conformation of the active site, any of these types of inhibition can occur, giving a possible explanation to the observed mixed type inhibition in vitro (Bapiro et al., 2001). The docking results also indicate that the observed TDI could arise from various mechanisms involving epoxidation of TBZ on the thiazole and benzyl rings. Further studies are therefore required to explore these potential biotransformations, results of which could result in modification of the metabolism scheme proposed in Fig. 1.

Assuming competitive inhibitory effects of TBZ on CYP1A2, SimCyp version 8.1 was used in the prediction of -fold increase in exposure of theophylline and caffeine (Table 2). Our result of 1.82-fold decrease in steady-state clearance of theophylline after TBZ therapy assuming competitive enzyme inhibition agrees with previously published clinical trial data (Schneider et al., 1990). Theophylline infusion was started 37 h after TBZ oral therapy, and they found 2.91-fold decrease in theophylline clearance; and from the pharmacokinetic principles (Lin and Pearson, 2002), the same magnitude in increase in drug exposure is anticipated. These findings are particularly important in the use of theophylline, a narrow therapeutic index drug, in which a small change in plasma concentrations can result in serious side effects (Lew et al., 1989; Schneider et al., 1990). With reference to other published literature (Schneider et al., 1990) and our findings, a dose reduction that is dependent on the route of administration and time of initiating the affected drug is recommended. The results for the effect of thiabendazole on caffeine are comparable with what has been found clinically for single doses of these two co-

![Fig. 12. Proposed routes by which thiabendazole is metabolized in vitro (a) and in vivo (b).](Image 1293INACTIVATION OF CYP1A2 BY THIABENDAZOLE 1293)
ingested drugs (Bapiro et al., 2005) for which an increase in AUC of 1.6 was observed.

Results from simulations of TDI effects on itself and those of theophylline and caffeine indicated that TBZ would inhibit its own clearance, resulting in >20-fold increase in exposure after only 3 days of administration (Table 2). The TBZ effects on theophylline and caffeine were predicted to result in 5.65 and 11.4 increases in exposure. These simulation results imply accumulation of TBZ with possible MBI activity on CYP1A2. The great impact of TBZ on its own elimination could partly be explained by it potentially being solely metabolized by CYP1A2 (fm1a2 = 1.0), whereas the drugs theophylline (fm1a2 = 0.85) (Monks et al., 1979) and caffeine (fm1a2 = 0.98) (Karjalainen et al., 2006) could be eliminated by other pathways.

Our MBI simulation results (Table 2) are contrary to some clinical reports that indicate that TBZ does not accumulate upon chronic administration (Schneider et al., 1990). In vitro results might offer an explanation for this poor in vitro to in vivo prediction of MBI-based DDI. In vitro studies using recombinant CYP1A2 clearly shows that TBZ is an MBI and that 5-OH TBZ is a weak inhibitor. The predicted pathway in vivo indicates the involvement of conjugation reactions that clear the 5-OH TBZ. We propose that in the in vitro system, in which there are no phase 2 reactions, 5-OH-TBZ accumulates and feedback inhibits this route. This leaves more TBZ available and increases the probability of the substrate docking (Fig. 11) mode associated with for bioactivation to metabolites associated with MBI (Fig. 12). This could explain why no MBI is observed in vivo because the MBI we observe is in vitro artifact because of the simplicity of the system devoid of other enzymes involved in TBZ metabolism and disposition. This could also be explained by the inherent differences between recombinant enzymes and human liver microsomes (Polasek and Miners, 2007).

The study was therefore able to show that TBZ is a potent MBI of CYP1A2. Although the use of computational methods as prediction tools for likelihood of TDI still needs to be validated, the study demonstrated they could be useful in both predicting and explaining observed characteristics in both in vivo and in vitro experiments. Future MBI studies will therefore be done using hepatocytes with proven UDP glucuronosyltransferase and sulfotransferase activity to verify our current hypothesis in explaining why no clinical MBI-based DDI have been observed.

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