IN VITRO AND IN SILICO IDENTIFICATION AND CHARACTERISATION OF THIABENDAZOLE AS A MECHANISM-BASED INHIBITOR OF CYP1A2 AND SIMULATION OF POSSIBLE PHARMACOKINETIC DRUG-DRUG INTERACTIONS

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Abbreviations: CYP: cytochrome P450; RMSD: root mean square distances; TDI: Time-dependent inhibition; MIC: metabolic intermediate complex; MBI: mechanism based inhibition; AUC: area under the curve; CL: clearance; TBZ: thiabendazole
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 $k_{\text{inact}}$ and $K_i$ of 0.08 and 0.02 min$^{-1}$ and 1.4 and 63.3$\mu$M 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 weak TDI of CYP1A2 and enzyme activity was recovered by dialysis. IC50 determination of TBZ and 50H-TBZ showed both compounds to potent inhibitors with IC50s of 0.83 and 13.05 $\mu$M 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 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. Predictions for MBI based DDI where however 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 where the proposed bioactivation route is minor. This might be the case for TBZ where, in vivo, UGTs and SUL, metabolise and eliminate the 5OH-TBZ.
Introduction

Thiabendazole (TBZ) is a broad-spectrum antihelminthic 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 post harvest treatment of fruit and vegetables and as a preservative in many consumer food products (Arenas and Johnson, 1994; Groten, et al., 2000; Szeto, et al., 1993; Walton, et al., 1999). Studies have shown that thiabendazole is extensively metabolised 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 catalysed hydroxylation to 5-hydroxythiabendazole which is further metabolised to glucuronide and sulphate conjugates (Coulet, et al., 1998). Other metabolites identified 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. TBZ has however 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 (Fujitani, et al., 1999; Mizutani, et al., 1990; Tada, et al., 1992), 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 (Bion, et al., 1995; Manivel, et al., 1987). Toxicity to the embryo has been associated with covalent binding of reactive metabolite from CYP 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 CYP. Covalent binding of metabolites to cellular macromolecules has been associated with drug toxicity and thiazole cleavage has been associated with toxicity via bioactivation by CYP to form epoxides (Mizutani, et al., 1994). It’s possible role and mechanisms of teratogenicity and hepatotoxicity in humans is 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 (Coulet, et al., 2000; Mizutani, et al., 1993). In rats, TBZ has also been shown to cause depletion of renal and hepatic GSH 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 CYP to form electrophilic species that can be trapped by GSH (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 \textit{in vitro} with a $K_i$ of 1.54$\mu$M (Bapiro, et al., 2001). Using an experimental set-up that evaluates reversible inhibition, the mode of inhibition was thought to be of mixed mechanisms.

Many of the observations in the earlier studies, for example covalent binding to protein, irreversible binding to tissue protein both \textit{in vivo} and \textit{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 characterised by time and concentration dependent loss of enzyme activity (Silverman and Daniel, 1995). MBI is a type of TDI where inhibition is permanent and restoration of activity is only by synthesis of new enzyme. MBI inactivate the CYP enzymes by either binding covalently to the heme, 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 if thiabendazole is a TDI using \textit{in vitro} methods and to identify likely sites of metabolism implicated in TDI \textit{in silico}. Potential drug-drug interactions via this mechanism were also simulated.
Materials and methods

Enzymes and bioanalytical reagents

Bactosomes prepared from *Escherichia coli* cells coexpressing recombinant human NADPH-P450 reductase and individual human P450s (CYP1A2, 2C9 and 3A4) were purchased from CYPEX (Dundee, UK). 3-Cyano-7-ethoxycoumarin (CEC), sulfaphenazole, ketoconazole, troleandomycin, thiabendazole, 5-hydroxythiabendazole, β-Nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt (NADPH), L-glutathione reduced and potassium ferricyanide were obtained from Sigma Chemical Co. (St. Louis, MO). 7-benzyloxy-4-trifluoromethylcoumarin (BFC), 7-methoxy-4-trifluoromethylcoumarin (MFC), furafylline and tienilic acid were obtained from GENTEST Corporation (Woburn, MA). Fluvoxamine was obtained from Tocris Bioscience (Ellisville, MO).

Crystal structure, hardware and software

Calculations were performed in a Linux (Red Hat 8.0) operating system on a 1.8 GHz Pentium IV. Software used included GRID version 22.2.2 and MetaSite version 2.7.5 (Molecular Discovery, http://moldiscovery.com), GOLPE version 4.5.12 (MIA, http://www.miasrl.com), SYBYL version 7.1 (Tripos Associates Inc., St. Louis, http://www.tripos.com) and CORINA (Molecular Networks GmbH, http://www.molnet.de). The energy calculations were performed using a script written in Perl language.

A crystal structure of CYP1A2 in complex with ANF (PDB2HI4) with a resolution of 1.95Å was used in this study. The enzyme is a wild type except that the terminal
transmembrane helical domain was removed to increase solubility for crystallisation. The inhibitor (α-naphthoflavone) was removed prior to docking. The structure of thiabendazole was drawn in Sybyl and converted to a 3-dimensional structure in CORINA. DDI simulations were done with Simcyp Version 8.1 (www.simcyp.com)

**In vitro experiments**

**TDI screen assay**

A two-step incubation scheme consisting of an inactivation assay and an activity assay was used. In the inactivation assay, TBZ (1 and 20µM) and 5OH-TBZ were incubated with appropriate concentrations of recombinant enzyme (Table 1) and phosphate buffer (0.1M pH 7.4). The assay was performed both in the presence and absence of NADPH for 15min at 37°C. A 10µl aliquot was then transferred to the activity assay consisting of substrate, phosphate buffer (0.1M pH 7.4) and NADPH (1mM). The reaction was then terminated by addition of an ice cold 20% Tris base / 80% ACN solution after 15 minutes. Activity of the enzyme was followed by measuring the formation of fluorescent metabolite. Appropriate controls were used in the assays on the effect of TBZ and 5OH-TBZ on CYP1A2 and TBZ on CYP2C9 and 3A4 activities (Table 1).

**IC₅₀ determination**

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 40µM) were preincubated both in the presence and absence of NADPH. The activity assay was then
performed and activity measured. The experiments were done with a high substrate concentration (CEC) of 12µM to minimise 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 0.31µM) were incubated with enzyme (0.5pmol/ well), KPO4 pH 7.4 (0.1M) and substrate at Km concentration (3µM). The reaction was initiated by the addition NADPH (1mM) and terminated by an ice cold 20% Tris base / 80% ACN solution after 15 minutes.

**Kinetics of CYP1A2 inactivation by thiabendazole**

The two-step incubation method was used to characterise the time and concentration dependent inhibition of CYP1A2 by TBZ and 5OH-TBZ. In the inactivation assay varying concentrations of TBZ (ranging between 0-20µM) and 5OH-TBZ (ranging between 1.56-200µM) were incubated with CYP1A2 (25pmol/ml), NADPH and phosphate buffer (0.1M 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 one in the TDI screen assay) and a further 15min incubation 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 (5mM), an electrophile-trapping agent. The glutathione was added together with TBZ (1 and 20µM) in 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 following oxidation by potassium ferricyanide (2mM in 0.1M 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 (50µl) was taken from the inactivation assay after 10 minutes and added to the restoration of activity plate, which had 50µl of the potassium ferricyanide (0.1M phosphate buffer pH 7.4 for the controls). After a further 10 minutes incubation another 10µl aliquot was taken and added to the activity assay plate consisting of fresh phosphate buffer (0.1M, pH 7.4), 1mM NADPH and 12µM 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 CHC.

**Effect of dialysis**

The two step assay was adapted to determine the effect of dialysis. In the inactivation assay CYP1A2 (25pmol/ml) was incubated with NADPH and either furafylline (1µM), fluvoxamine (20µM), TBZ (20µM), 5OH-TBZ (20µM) or 0.2% DMSO (vehicle control) in 0.1M phosphate buffer pH7.4. The incubation mixtures were transferred to Slide-A-Lyzer minidialysis units with a molecular weight cutoff of 10 000 ((Pierce Chemical Co.,
Rockford, IL.) Dialysis was performed at 4°C for 4 hrs in 1 Liter of 50 mM potassium phosphate buffer, pH 7.4. The dialysis buffer was changed every hour. Parallel analysis was done with incubation mixes which 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 normalised ratio**

In the screen assay the effect of each inhibitor on the activity of the enzyme were expressed as the normalised ratio calculated as shown in the equation. The classification was based on the method by Atkins and co-workers (Atkinson, et al., 2005)

\[
\text{Normalised ratio} = \frac{(R + I^{\text{NADPH}})/(R - I^{\text{NADPH}})}{(R + I^{\text{no NADPH}})/(R - I^{\text{no NADPH}})}
\]

where \(R + I^{\text{NADPH}}\) is the rate of reaction when incubation is performed in the presence of both inhibitor and NADPH, \(R - I^{\text{NADPH}}\) is the rate of reaction when incubation is performed in the presence of NADPH but in the absence of inhibitor, \(R + I^{\text{no NADPH}}\) is the rate of reaction when incubation is performed in the presence of inhibitor but in the absence of NADPH and \(R - I^{\text{no NADPH}}\) 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 lying between 0.7 and 0.9 fell in the grey-zone where their status cannot be clearly defined.
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**Determination of IC$_{50}$**

The amount of metabolite formed at each concentration relative to the control (% remaining activity) was plotted against the concentration of the inhibitor. A sigmoid curve was then fitted and IC$_{50}$ calculated using Prism (GraphPad Prism; GraphPad Software, San Diego, CA).

**Determination of $K_I$ and $k_{inact}$**

The natural logarithm of the percent remaining activity was plotted against the preincubation time at each inhibitor concentration to obtain the $k_{obs}$ (slope). The $k_{obs}$ is the rate constant describing the inactivation at each inhibitor concentration. Non-linear regression (GraphPad Prism; GraphPad Software, San Diego, CA) was then used to estimate the $k_{inact}$ and the $K_I$ from the equation below:

$$k_{obs} = \frac{k_{inact} \times [I]}{K_I + [I]}$$

where $k_{inact}$ is the maximal rate of inactivation, $K_I$ 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 prior to site of metabolism prediction to identify chemical groups in TBZ that are likely to cause TDI. An in-house script (Susanne Winiwarter, unpublished) 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 programme which 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 which 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 which is a GRID-based docking programme. The programme maps the active site using hydrophobic, hydrogen bond donor/acceptor and electrostatic probes. Prior to 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

Prior to docking, thiabendazole was built and energy minimised in vacuo using MMF94s force field and MMF94s charges using the conjugated gradient method. The active site
was defined with a radius of 15Å 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 optimise orientation of the ligand into the active site. During the optimisation the ligand was considered flexible and the enzyme active site rigid. Ten dockings were allowed with an early termination if the root mean square distances (RMSD) were within 1.5Å for the top three solutions.

**In silico drug-drug interactions (DDI): 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 thiabendazole’s pharmacokinetic and enzyme kinetic data. The Pharmacokinetics data of the CYP1A2 substrate drugs were already uploaded in the SimCyp software. In vivo co-administration of thiabendazole with CYP1A2 substrate drugs was only available for caffeine and theophylline. This was followed by modelling where we mimicked published experimental methodology (dose, interval, duration, sample size, etc) 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 DDI tested and an additional theophylline-thiabendazole interaction was also simulated following theophylline infusion (Schneider, et al., 1990).
Competitive inhibition

In these simulations, thiabendazole was the competitive inhibitor with a $K_i$ of $1.54 \mu M$ 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.

Mechanism based inhibition (MBI)

Clinical drug doses, frequency and duration of three days (thiabendazole is dosed twice daily for three days) were uploaded at trial design stage. Thiabendazole’s effect 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 fig 2 and 3 thiabendazole was a clear TDI for CYP1A2, but not for CYP2C9 and was unclear for CYP3A4. 5OH-TBZ was a weak TDI for CYP1A2 as indicated by the normalised 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 where 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 of 0.83µM and 13.05µM 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 when preincubated with NADPH. Since TBZ had been shown to be a potent TDI in the TDI assay above the assay was used to further confirm the observed result. TBZ inhibited CYP1A2 activity with an IC₅₀ value of 84.5µM 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 2.8µM.

**Kinetics of CYP1A2 inactivation by TBZ and 5OH-TBZ**

Kinetics of CYP1A2 inactivation by TBZ and 5OH-TBZ were followed by measuring loss of CEC dealkylation activity (Fig 6a 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. Non-linear 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, $k_{\text{inact}}$ and $K_I$ for CYP1A2 were determined to be $0.08 \text{min}^{-1}$ and $1.4 \mu\text{M}$ respectively for TBZ and $0.02 \text{min}^{-1}$ and $63.03 \mu\text{M}$ for 5OH-TBZ respectively. At higher concentrations of thiabendazole, some loss of activity were observed at the zero point due to the carryover of thiabendazole into the inactivation assay mixture. Since 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 6.3µM). However the changes were much clearer at the higher concentrations giving much 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 (Fig 7). 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 CYP1A2 as described in materials and methods. For comparison, the experiment was also conducted with samples that were not dialysed. 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 as there was no significant restoration of activity after dialysis. 5OH-TZ 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 metabolised to reactive metabolites that bind irreversibly to the enzyme (Fontana, et al., 2005). The compound was then submitted to Metasite which is a programme that predicts the likely site of metabolism. The programme 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 which 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 where the benzene ring of the compound was the 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). In GLUE eight of the ten docking solutions were in agreement with the site of metabolism prediction. In five of the ten solutions the thiazole ring was towards 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 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 to the competitive effects for the CYP 1A2 substrate tested.
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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 which might be associated with toxic effects. In this study TBZ was screened for potential TDI against CYP2C9 and CYP3A4 only based on the fact that a significant number of compounds have shown TDI against these enzymes. The choice of CYP1A2 was based on the fact that TBZ had 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 Ki determined for TBZ (1.4µM) was lower than the total plasma concentrations the drug is capable of reaching (19µM) (Bapro, et al., 2005). The k_inact of 0.08min⁻¹ is relatively high such that the resulting k_inact/Ki of 0.05µM/min is predictive of likely significant enzyme inactivation. In the treatment of strongyloidiasis in humans, the drug is given over 3 days at 25mg/kg/day (Merck, 1999; Satoh and Kokaze, 2004) thus providing the time component during which CYP1A2 will progressively be inactivated. In a chronic exposure study to thiabendazole (50mg/kg/day), the pharmacokinetic studies did not however show accumulation of thiabendazole (Bauer, et al., 1982).
TBZ showed much lower potency when preincubated in the absence (IC50 = 84.5 µM) compared to the presence of NADPH (IC50 = 2.8 µM) (Fig 5). This shift in the IC50 further suggests that TBZ is a likely TDI. It should be noted however that the high value (84.5 µM) cannot be compared to the low IC50s (1.2 and 0.83 µM) obtained in the reversible inhibition assay (Bapiro, et al., 2001) since the inhibitor is diluted ten-fold before the inhibition is allowed to occur. A high substrate concentration was used to minimise competitive inhibition, hence the high IC50 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 prior to inactivation. This is one of the characteristics that differentiate irreversible inhibitors from the reversible ones. In the case of irreversible inhibitors glutathione will have no effect in preventing inactivation since 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 following 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, fluvoxamine. 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) where 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 out of 10 docking poses in GLUE showed the site at a favourable distance to the heme catalytic center. The thiazole group was predicted as the likely site of metabolism by both Metasite and five out of ten 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., 2005; Bapiro, et al., 2001). The docking results could explain the observed results. When the compound is docked with the benzyl moiety oriented towards the heme (Fig 11a) then hydroxylation to 5-hydroxythiabendazole which is the main route of metabolism is favoured. The docking solution could explain the competitive inhibitory effects of thiabendazole on CYP1A2. When the thiazole moiety docks close to the heme (Fig 11b) then 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), then non-competitive 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 \textit{in vitro} \cite{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 \cite{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 \cite{Schneider, et al., 1990}. Theophylline infusion was started 37 hours after TBZ oral therapy and they found 2.91 fold decrease in theophylline clearance, and from the pharmacokinetic principles \cite{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, where a small change in plasma concentrations can result in serious side effects \cite{Lew, et al., 1989; Schneider, et al., 1990}. With reference to other published literature \cite{Schneider, et al., 1990} and our findings, a dose reduction which is dependent on the route of administration and time of initiating the affected drug is recommended. The results for thiabendazole’s effect on caffeine are comparable with what has been found clinically for single doses of these two co-ingested drugs \cite{Bapiro, et al., 2005} were 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 over 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 the possible MBI activity on CYP1A2. The great impact of TBZ on its own elimination could partly be explained by the fact that it might be solely be metabolised by CYP1A2 ($f_{m1A2}=1.0$) whereas the other drugs, theophylline ($f_{m1A2}=0.85$) (Monks, et al., 1979) and caffeine ($f_{m1A2}=0.98$) (Karjalainen, et al., 2006) could be eliminated by other pathways.

Our MBI simulation results (Table 2), are contrary to some clinical reports which indicate that TBZ does not accumulate upon chronic administration (Schneider, et al., 1990). Our 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 a MBI and that 5-OH TBZ is a weak inhibitor. The proposed pathway $in vivo$ indicates the involvement of conjugation reactions that clear the 5OH TBZ. We propose that in the $in vitro$ system, where there are no phase two reactions, the 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 $in vivo$ since the one we observe is an in vitro artefact due to 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 HLM (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 need 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 UGT and sulfanotransferase activity in order to verify our current hypothesis in explaining why no clinical MBI based DDI have been observed.
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Footnotes

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Figure legends

Figure 1: Proposed biotransformation of TBZ (its elimination and bioactivation) (Coulet, et al., 2000; Dalvie, et al., 2006; Fujitani, et al., 1991; Mizutani, et al., 1994)

Figure 2: Effect of TBZ and 5OH-TBZ on the activity of CYP1A2. Furafylline and fluvoxamine (0.1 and 1µM) were used as controls as described under Materials and Methods.

Figure 3: Effect of thiabendazole on CYP3A4 and CYP2C9 activity. Known time dependent inhibitors (TDI) (tienilic acid and trolendomycin for CYP2C9 and CYP3A4 respectively) and non-TDI (sulfaphenazole and ketoconazole) for each of the isoforms were used as positive and negative controls respectively. High and low concentration for thiabendazole were 1 and 20µM and for the controls 0.1 and 1µM. The normalised ratio was calculated as described in Materials and Methods.

Figure 4: Inactivation of CYP1A2 mediated CEC metabolism by thiabendazole in the presence and absence of NADPH in the preincubation step.

Figure 5: Inhibition of the recombinant CYP1A2-catalyzed fluorometric reaction by TBZ and 5OH-TBZ.

Figure 6: Time and concentration dependent inactivation of cyano ethoxy-coumarin (CEC) dealkylation by TBZ and 5OH-TBZ. CYP1A2 was preincubated with varying concentrations of thiabendazole, aliquots (10µl) removed at time points indicated and
assayed for residual activity as described under Materials and Methods (a). The $k_{obs}$ was then determined for each concentration and data fitted using non-linear regression to obtain $k_{\text{inact}}$ and $K_I$ (b).

*Figure 7: Effect of glutathione and potassium ferricyanide on the inactivation of CYP1A2 by thiabendazole.* Thiabendazole was incubated with glutathione (5mM) and potassium ferricyanide (2mM) and CYP1A2 CEC dealkylase activity determined as described under Materials and Methods. The data shown is a mean of two independent experiments.

*Figure 8: Effect of dialysis on inactivation of CYP1A2 CEC dealkylase activity by TBZ and 5OH-TBZ.* CYP1A2 (25pmol/ml) was preincubated with 0.2% DMSO (vehicle control), furafylline, fluvoxamine, TBZ and 5OH.TBZ as described under materials and methods. A 0.5ml aliquot was transferred to a SLIDE-A-LYSER mini dialysis unit, and dialysed for 4hrs prior to determination of activity. Parallel analysis was conducted in which they was no dialysis. Activity was then assayed as described under Materials and Methods.

*Figure 9: Site of metabolism prediction and substructure search in thiabendazole.* Two fragments associated with TDI, the thiazole moiety and a conjugated system were identified (a) The structure and numbering of thiabendazole is also shown and the known hydroxylation site (from literature) is circled. The site of metabolism was then predicted using MetaSite (b). Top three sites of metabolism predicted by the programme are indicated by arrows with the position ranked first circled.
Figure 10: Binding of thiabendazole into the active site of CYP1A2 (PDB: 2HI4). Docking experiment and analysis of receptor ligand interaction were done by GOLD as described under materials and methods. The heme prosthetic group, amino acid residues constituting the active site and thiabendazole are all depicted in sticks. Carbon atoms are coloured orange in thiabendazole, and green in the heme and amino acid residues. Oxygen, nitrogen and heme iron are coloured red, blue and purple respectively. Hydrogens and water molecules are not shown.

Figure 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 colour coding is the same as described for legend 7. In five of the top ten ranked solutions, the thiazole group was the group closest to the heme (b), in three the benzene ring where 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.

Figure 12: Proposed routes by which thiabendazole is metabolised in vitro(a) and in vivo(b)
## Tables

### Table 1: Experimental conditions for the screen assay

<table>
<thead>
<tr>
<th>CYP</th>
<th>a Enzyme amount (pmol/ml)</th>
<th>Substrate</th>
<th>b Substrate concentration (µM)</th>
<th>Controls</th>
<th>Excitation wavelength</th>
<th>Emission wavelength</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A2</td>
<td>25</td>
<td>CEC</td>
<td>12</td>
<td>Furafylline Fluvoxamine</td>
<td>405</td>
<td>460</td>
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<tr>
<td>2C9</td>
<td>300</td>
<td>MFC</td>
<td>250</td>
<td>Tienilic acid Sulfaphenazole</td>
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<td>535</td>
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<tr>
<td>3A4</td>
<td>50</td>
<td>BFC</td>
<td>13</td>
<td>Troleandomycin Ketoconazole</td>
<td>405</td>
<td>535</td>
</tr>
</tbody>
</table>

CEC, 3-cyano-7-ethoxycoumarin; MFC, 7-methoxy-4-trifluoromethylcoumarin; BFC, 7-benzyloxy-4-trifluoromethylcoumarin.

a the enzyme amount in the preincubation mix (10x required in second incubation)

b substrate concentration at 4x the Km (to reduce the effect of reversible inhibition)
<table>
<thead>
<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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<tbody>
<tr>
<td>Thiabendazole/Thiabendazole</td>
<td>MBI</td>
<td>22.51</td>
<td>1.0</td>
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<tr>
<td>Thiabendazole/Caffeine</td>
<td>Competitive</td>
<td>1.23</td>
<td>1.6</td>
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<td>2.91</td>
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<tr>
<td>Thiabendazole/Theophylline</td>
<td>MBI</td>
<td>5.65</td>
<td></td>
</tr>
</tbody>
</table>
Figure 1

4-hydroxyTBZ → Thiabendazole (TBZ) → 5OH-TBZ glucuronide

4-acetylbenzimidazole → Thiabendazole (TBZ) → 5-hydroxyTBZ

5OH-TBZ glucuronide → 5OH-TDZ sulfate

GSH → DETOXIFICATION

Dihydroxythiabendazole → epoxide → diol

Benzimidazol-2-yl glyoxal + thioformamide → NEPHROTOXICITY

Benzimidazol-2-yl glyoxal + protein -NH₂ → COVALENT BINDING
Figure 2

![Bar chart showing normalized ratios for different compounds. The x-axis represents different compounds: furafylline, TBZ, 5OH-TBZ, and fluvoxamine. The y-axis represents the normalized ratio, ranging from 0.1 to 1.3. The chart includes error bars for low and high concentrations. The compounds are categorized into non-TDI, grey zone, and clear TDI.](image-url)
Figure 3

CYP3A4

<table>
<thead>
<tr>
<th>Compound</th>
<th>Low Conc.</th>
<th>High Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ketoconazole</td>
<td>1.1</td>
<td>1.5</td>
</tr>
<tr>
<td>Troleandomycin</td>
<td>0.5</td>
<td>0.7</td>
</tr>
<tr>
<td>Thiabendazole</td>
<td>0.9</td>
<td>0.7</td>
</tr>
</tbody>
</table>

CYP2C9

<table>
<thead>
<tr>
<th>Compound</th>
<th>Low Conc.</th>
<th>High Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfaphenazole</td>
<td>1.3</td>
<td>1.1</td>
</tr>
<tr>
<td>Tienilic acid</td>
<td>0.9</td>
<td>0.7</td>
</tr>
<tr>
<td>Thiabendazole</td>
<td>0.9</td>
<td>0.7</td>
</tr>
</tbody>
</table>
Figure 5

The graph shows the effect of Thiabendazole concentration on enzyme activity in the presence and absence of NADPH. The x-axis represents the concentration of Thiabendazole in μM, ranging from 0.01 to 100. The y-axis represents the percentage of remaining activity. The data points indicate a significant decrease in activity as the concentration of Thiabendazole increases. The addition of NADPH shifts the curve to the left, suggesting an increase in sensitivity to Thiabendazole.

The x-axis is labeled as [Thiabendazole] (μM), and the y-axis is labeled as % remaining activity.
Figure 6

(a) Graph of ln% remaining activity vs. time for Thiabendazole at different concentrations.

(b) Graph of k_{obs} (min^{-1}) vs. [Thiabendazole] (µM) and [5-hydroxythiabendazole] (µM).
Figure 7

![Bar chart showing remaining activity (% of control) for different treatments.](chart.png)

- Thiabendazole
- Thiabendazole + GSH
- Thiabendazole + K₃FeCN₆
- Thiabendazole + GSH + K₃FeCN₆

The chart compares the remaining activity of thiabendazole with and without NADPH in different combinations of treatments.
Figure 10

The diagram shows a molecular structure with various amino acids labeled, including PHE-260, PHE-256, PHE-226, VAL-227, THR-223, ASP-320, THR-321, LEU-382, ILE-386, THR-124, and ALA-317. Distances are marked, such as 4.37 Å and 4.54 Å, indicating interactions between the molecules. The structure also includes a labeled heme group.