Knowledge of how drugs are metabolized and excreted is an essential component of understanding their fate within and among target and non-target organisms. Thiabendazole (TBZ) was the first benzimidazole (BZ) to be commercially available and remains one of the most important anthelmintic drugs for medical and veterinary use. We have characterized how *Caenorhabditis elegans* metabolizes and excretes TBZ. We have shown that TBZ directly binds to the nuclear hormone receptor (NHR)-176 and that this receptor is required for the induction by TBZ of the cytochrome P450 (CYP) encoded by cyp-35d1. Further, RNAi inhibition of cyp-35d1 in animals exposed to TBZ causes a reduction in the quantity of a hydroxylated TBZ metabolite and its glucose conjugate that is detected in *C. elegans* tissue by HPLC. This final metabolite is unique to nematodes and we also identify two P-glycoproteins (PGPs) necessary for its excretion. Finally, we have shown that inhibiting the metabolism we describe increases the susceptibility of *C. elegans* to TBZ in wild-type and in resistant genetic backgrounds.

**Key words:** *Caenorhabditis elegans*, cytochrome P450 (CYP), excretion, metabolism, nuclear hormone receptor (NHR), P-glycoprotein (PGP).

**INTRODUCTION**

In humans, oxidation reactions by cytochrome P450 (CYP) enzymes play an important role in the metabolism of a vast number of endogenous and exogenous compounds. This superfamily is responsible for the bio-transformation of approximately 90% of commonly used pharmaceuticals [1]. Detoxification pathways that metabolize and excrete endobiotic/exobiotic molecules are typically described as acting in three successive phases. CYPs are major enzymes involved in oxidation reactions in phase I cellular metabolism and are often required to render molecules suitable substrates for phase II metabolism. The conjugation reactions that occur in phase II metabolism are catalysed by GSTs and UDP-glycosyltransferases (UGTs). The resulting soluble metabolites are then excreted by multi-drug efflux pumps, including ATP-binding cassette (ABC) transporters, during phase III. The human genome contains 57 predicted CYPs, divided among 18 families and 44 subfamilies [2]. Only three of these families (CYP1, CYP2 and CYP3) are responsible for drug and steroid metabolism, with CYP3A and especially CYP3A4 being the most important in humans [3].

Although bio-transformation pathways of pharmaceuticals have been extensively studied in mammals, the response and extent to which these compounds are metabolized in other target and non-target animals has been less well-studied. Analysis of genome sequence data from invertebrates reveals the presence of all the key enzyme families known to catalyse bio-transformation in animals. Furthermore, an expansion in the CYP superfamily is present in insects and nematodes with 90 CYPs being predicted in *Drosophila* [4] and 83 in *Caenorhabditis elegans* [5]. Earlier studies have shown that some members of cellular metabolism gene families, particularly the CYPs, are highly inducible in *C. elegans* by a range of small organic molecules [6–8]. We have recently shown that members of the CYP family are also induced by thiabendazole [TBZ; 2-(4-thiazolyl)-1H-benimidazole] in *C. elegans* and that the most inducible CYP with the highest expression and for the longest duration is regulated by *nhr-176* [9]. Bio-transformation of this commonly used anthelmintic has been studied in mammals. TBZ is extensively metabolized via hydroxylation of the benimidazole (BZ) ring at the 5-position to form 5-hydroxythiabendazole, which is further metabolized to glucuronide and sulfate conjugates [10,11]. TBZ induces the expression of CYP families CYP1A and CYP2B in rats *in vivo* [12] and rabbit CYP1A2 *in vitro* [13]. Members of CYP1A and CYP2B families in mammals are regulated by the nuclear hormone receptors (NRHs), pregnane X receptor (PXR) and constitutive androstane receptor (CAR) [14]. NRHs act as transcription factors for metabolic genes and are functionally activated by ligand binding. Xenobiotic, as well as endobiotic, molecules can act as ligands and this can allow dynamic changes in gene expression following the entry of new chemicals within the organism. The selectivity of NRHs is not well understood making it challenging to assess how many potential ligands an NHR might have, but mammalian PXR in particular displays high promiscuity for ligand binding [15].

Parasitic nematodes of humans and livestock cause diseases of major socio-economic importance worldwide. Three main classes of synthetic chemicals have been commonly used to treat gastrointestinal helminths; BZ, the macrocyclic lactones (ML) and the imidazothiazoles/tetrahydropyrimidines (LV). TBZ was the first BZ to be marketed and is used as a broad-spectrum anthelmintic drug for medical and veterinary use as well as in agriculture. Although bio-transformation of TBZ has been extensively studied in mammals, the response and extent to which these compounds are metabolized in non-target and non-target organisms remains unknown. TBZ is metabolized by phase I and II metabolism pathways to form five major metabolites and is then excreted in the urine of treated animals. These bio-transformed metabolites are unique to nematodes and some have been shown to be species-specific [16]. The identification of the enzymes responsible for TBZ metabolism in *Caenorhabditis elegans* will provide new insights into the metabolism of anthelmintics in non-target organisms.

**Abbreviations:** ABC, ATP-binding cassette; ABZ, albendazole; BHT, dibutyl hydroxytoluene; BZ, benzimidazole; CAR, constitutive androstane receptor; CYP, cytochrome P450; ITC, isothermal titration calorimetry; NGM, nematode growth medium; NHR, nuclear hormone receptor; PGP, P-glycoprotein; PPAR, peroxisome-proliferator-activated receptor; PXR, pregnane X receptor; Q-PCR, quantitative PCR; TBZ, thiabendazole; TOF, time-of-flight; UGT, UDP-glycosyltransferase.

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an anthelmintic to treat parasitic infections in humans and veterinary animals [16]. It has also been used as an agricultural fungicide for pre- and post-harvest treatment of vegetables and fruits. Its continued use since the 1960s has consequently led to numerous reports of resistance in livestock [17] and in fungi [18]. As is well known in pharmaceuticals, knowledge of the key interactions between a drug and the organisms it acts upon can enable drug discovery and assist in effective resistance management. Metabolism of pesticides in insects has an important effect on drug potency and plays a key role in insecticide resistance in a number of cases [19–22]. Research using C. elegans as a model organism has facilitated the identification of the protein target of the BZs. Twenty-eight mutations which confer BZ resistance on C. elegans have been identified and all map to a single locus, ben-1, that encodes β-tubulin [23]. This suggests that the ben-1-encoded β-tubulin in C. elegans is required for sensitivity to BZs. Similar results have subsequently been observed in resistant isolates of parasitic species, with point mutations between codons 128 and 200 in β-tubulin genes being associated with resistance [24–30]. Taken together, these results suggest that BZs affect nematodes by interacting with β-tubulin.

The work presented in the present paper extends these studies by considering the metabolism and excretion of BZs in vivo, a process which affects the availability of BZs within treated nematodes and therefore together with the β-tubulin interaction, is a determinant of BZ toxicity. The present study aims to identify key interactions between TBZ and nematode metabolism/excretion and the consequences of these interactions on the potency of TBZ. First, the ability of recombinantly expressed NHR-176 to bind TBZ was determined using isothermal titration calorimetry (ITC). Quantitative PCR (Q-PCR) was used to assess the effect of targeting nhr-176 and cyp-35d1 by RNAi on the expression of genes induced by TBZ across a 6 h time course. HPLC–UV analysis of metabolites extracted from nematodes was used to deduce the effect of knocking-down cyp-35d1 on TBZ metabolism. Susceptibility to TBZ was also investigated in P-glycoprotein (Pgp; ABC transporter) deletion strains. Finally, the effect of reducing transcript for cyp-35d1 and its transcription factor on susceptibility to TBZ in wild-type and ben-1-encoded β-tubulin deletion backgrounds was determined.

MATERIALS AND METHODS

Isothermal titration calorimetry (ITC)

Recombinant NHR-176 was expressed using the pET28a/BL21 (DE3) system and purified using 1 ml of histidine–Trap FF column and an AKTA Explorer (GE Healthcare). ITC experiments were carried out at 25°C on a VP–ITC calorimeter from MicroCal. Recombinantly expressed NHR-176 and BSA (Sigma–Aldrich) were dialysed in 10 mM Tris/HCl, 150 mM NaCl and 0.01% Triton X-100, pH 7.5. Both the proteins were dialysed in 5 ml volumes at 4°C against 1 litre of buffer, which was changed three times during a 36-h period. The samples and buffer were pretreated using vacuum filtering and degassing for 20 min before the ITC experiment. The sample cell was filled with 2 ml of rNHR-176 titrant and titrated against TBZ, which was filled in the syringe of 300 μl at 400 μM. The concentrations of rNHR-176 and TBZ were 0.01 mM and 0.1 mM respectively. TBZ was dissolved in a final concentration of 0.4% DMSO. The injections were performed using a volume of 10 μl per injection, 20 s for the duration of the injection and with a 150 s interval between the injections. The reaction mixture was continuously stirred at 300 rev./min during the titration. Control experiments were carried out by injecting TBZ and DMSO into buffer as well as DMSO only into protein under the same conditions to take the heats of dilution and viscous mixing into account. The heats of injection of the control experiment were subtracted from the raw data of rNHR-176 titration. The ITC data were analysed using the ORIGIN version 7.0 software provided by MicroCal.

The heats of binding were normalized with respect to the titrant concentration and a volume correction was performed to take into account dilution of titrant during each injection. The amount of heat produced per injection was calculated by integration of the area under each peak using a baseline selected by the ORIGIN program, assuming a one-site binding model. The dissociation constant (Kd) and molar enthalpy (ΔH) for the binding of TBZ to rNHR-176 were determined by non-linear least square fitting to the data.

RNAi liquid culture and Q-PCR analyses

Mixed stages of C. elegans wild-type strain N2 (Bristol) were cultivated in liquid S Basal medium [0.1 M NaCl, 0.05 M potassium phosphate, pH 6, and 5 μg ml−1 cholesterol (from a 5 mg ml−1 stock in ethanol)] supplemented with 50 μg ml−1 nystatin (Sigma–Aldrich), 50 μg ml−1 streptomycin (Sigma–Aldrich) on a diet of Escherichia coli HB101, as described previously [31]. Following treatment with sodium hypochlorite of the mixed stage culture approximately 106 arrested L1s were added to 6 × 300 ml conical flasks and incubated at 20°C on an orbital incubator (Beckman) operating at 200 rev./min. RNAi knockdown was performed in liquid culture as described previously [32]. Nematodes were fed freshly harvested HT115 (DE3) cells containing pl4440 clones targeting cyp-35d1, nhr-176 and gfp for 72 h daily prior to treatment with 0.5 mM TBZ. Nematodes were harvested at 1.5, 3.0, 4.5 and 6.0 h following TBZ exposure and were separated from E. coli and debris by sucrose floatation, washed with chilled S basal and flash frozen in liquid nitrogen. RNA was extracted using an RNAeasy kit (Qiagen) and cDNA was prepared using Superscript II Reverse Transcriptase (Invitrogen). The expression of cyp-35d1 and nhr-176 was measured following DMSO and TBZ exposure of nematodes fed dsRNA targeting both these genes. In order to investigate the regulation of other CYP genes by nhr-176 or any RNAi off-target effects the expression of cyp-35a3, cyp-35a5 and cyp-35d1 was also assessed. Two stable transscripts (ama-1 and Y35g12.2) were used as normalizing genes and the oligonucleotide sequences of the primers are given in Supplementary material. Brilliant III Ultra-Fast SYBR® Q-PCR Master Mix (Agilent) was used without additional magnesium. The Bio-Rad CFX96 was programmed as follows; 3 min at 95°C followed by 40 cycles of 5 s at 95°C and 10 s at 60°C.

Extraction of TBZ and its metabolites

TBZ and metabolites were extracted in 200 μl of saturated sodium hydroxide carbonate, pH 10, 200 μl of dibutyl hydroxytoluene (BHT; 100 μg of BHT in 10 ml of acetonitrile) and 6 ml of acetonitrile. Packed frozen nematodes (1 ml) were homogenized on ice using 3 × 30 s cycles with 1 min intervals of sonication (maximum amplitude, 16 μm) with a Soniprep 150 (Sanyo). The samples were centrifuged at 3500 g at 4°C for 10 min to remove solid debris, added to an equal volume of n-hexane and shaken for 5 min at 25°C. The acetonitrile layer was collected and evaporated to dryness at 40°C using an EZ-2 (Genevac). Dried residues were resuspended in 400 μl of acetonitrile/methanol/0.02 M ammonium formate, pH 3.3 (2:2:6, by vol) and insoluble metabolites were removed by centrifugation at 10000 g at 4°C for 15 min. For determination of extraction...
efficiency of TBZ and TBZ-OH, 1 ml of packed nematodes were spiked with 0.02, 0.25, 1.25 and 2.50 μg·mL⁻¹ of standard dissolved in acetonitrile/methanol/0.02 M ammonium formate, pH 3.3 (2:2:6, by vol.) from a 1 mg·mL⁻¹ stock dissolved in DMSO prior to extractions. For extraction of TBZ and metabolites from culture medium, 5 ml of culture medium was added to 1000 μl of saturated sodium hydrogen carbonate, pH 10, 1000 μl of BHT (100 μg in 10 ml of acetonitrile) and 30 ml of acetonitrile and extractions were carried out in the same way as from nematodes.

HPLC and MS/MS analyses
Reverse-phase HPLC analysis was carried out using a Dionex Summit (Dionex) and a Cosmosil 5C₁₈ MS-II column (5 μm, 4.6 mm internal diameter × 250 mm; Nacals Tesque) according to adaptations of an established method [33]. Photodiode array detection was used with a scan range of 200–400 and UV detection was set at 290 nm and 320 nm. Acetonitrile (A) and 0.02 M ammonium formate, pH 3.3 (B) were used as the mobile phase with a linear gradient profile which started at 10% A, increased to 70% A by 19 min, then reduced to 10% from 19 to 21 min. The flow rate was 1 ml·min⁻¹ and injection volumes were 380 μl. Fractions were collected every minute and data were acquired and processed using Chromleven version 6.50. Peaks were manually identified and relative peak areas were exported to Microsoft Excel 2010 for statistical analysis. Fractions of 2 ml were collected and reconstituted in 50 μl of saturated sodium hydrogen carbonate, pH 10, 1000 μl of DMSO prior to extractions. For extraction of TBZ and metabolites from culture medium, 5 ml of culture medium was added to 5 ml of saturated sodium hydrogen carbonate, pH 10, 1000 μl of BHT and 30 ml of acetonitrile and extracted with organic solvent.

Assessment of susceptibility to TBZ and TBZ-OH under different genetic backgrounds and RNAi
Susceptibility to TBZ was investigated for pgp deletion strains NL132 pgp-1(pk17) IV, GH378 pgp-2(kx48) I, NLI31 pgp-3(pk18) X, VC2159 pgp-4(gk1006) X, RB959 pgp-5(ok856) X, RB1047 deletion in pgp-7 and pgp-6(ok994) X, RB1916 pgp-8(ok2489) X, pgp-9 (tm0830), RB1045 pgp-10(ok991) X, pgp-11 (tm0333) VC26 pgp-12(gk19) X, RB894 pgp-13(ok747) X, RB2008 pgp-14(ok2660) X and RB1041 pgp-15(ok987) X. C. elegans wild-type Bristol N2 strain was used as a negative control. Assays were carried out on 48-well plates containing NGM (nematode growth medium) with the appropriate concentration of TBZ dissolved in DMSO to a final concentration of 1%. Plates were seeded with HB101(DE3) and bacterial lawns were allowed to grow overnight at 25°C before approximately ten arrested L1 stage C. elegans N2 wild-type or pgp deletion mutants were transferred to individual wells. Following 3 days of incubation at 25°C, the numbers of progeny in each well were counted. Each well culture was replicated at least three times. Assessment of CB3474 ben-1(e1880) III mutant susceptibility to TBZ and TBZ-OH knockdown of cyp-35d1 and nhr-176 by RNAi was achieved by feeding on plates as described previously [32]. E. coli HT115(DE3) clones targeting gfp and nhr-176 were obtained commercially from Source Bioscience (http://www.lifesciences.sourcebioscience.com) and grown overnight in LB liquid containing 50 μg·ml⁻¹ ampicillin. From each culture, 5 μl was then seeded directly into 48-well plates containing NGM. NGM contained 1 mM IPTG, 50 μg·ml⁻¹ ampicillin and the appropriate concentration of TBZ or TBZ-OH dissolved in DMSO to a final concentration of 1%. Bacterial lawns were allowed to grow overnight at 25°C and 10 ± 1 arrested L1 stage C. elegans N2 wild-type or CB3474 ben-1(e1880) III mutants were transferred to individual wells. Following 3 days of incubation at 25°C, the numbers of progeny in each well were counted. Each well culture was replicated at least three times. Comparisons were made to the appropriate N2 wild-type and gfp controls using a Student’s t test which assumed a two-tailed distribution and two-sample unequal variance (heteroscedastic).

RESULTS
Recombinant NHR-176 binds TBZ
We have shown previously that nhr-176 is required for induction of cyp-35d1 by TBZ [9]. This may be because NHR-176 binds and is activated by TBZ directly or that NHR-176 acts downstream of another protein that interacts with TBZ. To distinguish these possibilities we determined whether or not recombinant NHR-176 (rNHR-176) binds to TBZ by ITC. The results indicate that rNHR-176 is able to bind TBZ under physiologically relevant conditions. The ITC titration of rNHR-176 with TBZ revealed a 1:1 stoichiometry and dissociation constant (K_d) values of 4.8 μM for TBZ, with ΔH of −2.897 × 10⁻² ± 259.2 cal·mol⁻¹ (1 cal≈4.184 J), ΔS of 14.61 cal·mol⁻¹·deg⁻¹, TΔS of 24.887 × 10⁻³ cal·mol⁻¹ and ΔG of −27.784 × 10⁻³ cal·mol⁻¹. This indicates an exothermic reaction in which rNHR-176 binds TBZ as a monomer.

NHR-176 regulates expression of cyp-35d1
Q-PCR was used to determine the expression of TBZ inducible CYPs when transcripts for nhr-176 and cyp-35d1 were individually targeted by RNAi prior to TBZ exposure. The expression of cyp-35a3, cyp-35a5 and cyp-35c1 was increased up to 100-fold (Figure 1) after 1.5 h TBZ exposure in gfp controls compared with those exposed to DMSO, but not as strongly as that of cyp-35d1. Following 1.5 h of TBZ exposure the relative expression of cyp-35d1 was increased from < 0.05 to 105 (>2000-fold) in the gfp control and had declined to 44 and 18 after 3 h and 6 h respectively (Figure 1). The expression of nhr-176 was not affected by TBZ exposure across any time-points in the gfp control. However, when nhr-176 was targeted by RNAi, the expression of cyp-35d1 was knocked down to approximately 37 ± 8% after DMSO exposure and to approximately 17 ± 3% after TBZ exposure compared with the gfp control. In other words the induction of cyp-35d1 is ablated in an nhr-176 (RNAi) background. When cyp-35d1 was targeted directly by RNAi, the expression of this gene was knocked down to 40 ± 10% under DMSO exposure and to 15 ± 4% under TBZ exposure compared with the gfp control, which is similar to the effect of knocking down nhr-176. The expression of cyp-35a3, cyp-35a5 and...
CYP-35D1 catalyses the oxidative metabolism of TBZ

Having shown that TBZ binds to NHR-176 and that this is required for increased expression of cyp-35d1, we then measured whether or not CYP-35D1 catalysed the oxidation of TBZ. Transcript for cyp-35d1 was knocked down by RNAi and the abundance of TBZ and its metabolites was measured using HPLC–UV. TBZ was recovered from metabolite extractions from 1 ml of packed nematodes spiked with 0.02, 0.10 and 0.50 p.p.m. of the standard with an efficiency of 108.56 ± 11.46 %. TBZ-OH was recovered from extractions spiked with 0.10 and 0.50 p.p.m. of the standard with an efficiency of 108.56 ± 11.96 %. However, recovery of TBZ-OH from extractions spiked with 0.02 p.p.m. of the standard was impossible to quantify. TBZ had an elution time of approximately 11 min and TBZ-OH of approximately 8 min (Supplementary Figure S1). Peaks representing both TBZ and TBZ-OH were identified in all metabolite extractions from RNAi targeted nematodes across the 6 h time course and were confirmed by MS/MS fragmentation. Along with a metabolite which eluted at approximately 5 min, these were the most abundant peaks. Fragmentation of TBZ typically shows three major fragment ions with masses of 202.05, 175.04 and 131.07 Da, whereas fragmentation of TBZ-OH shows three major fragment ions with masses of 218.05, 191.04 and 147.06 Da (Supplementary Figure S2).

A significantly greater abundance of TBZ (as calculated from relative peak area) was found when transcript for cyp-35d1 was targeted by RNAi compared with gfp controls across the 6 h time course (P < 0.05), whereas the abundance of TBZ-OH was significantly lower with gfp controls (P < 0.01; Figures 2a and 2b). This is consistent with CYP-35D1 catalysing the formation of TBZ-OH. Furthermore, the abundance of a metabolite that eluted at approximately 5 min was also significantly reduced in nematodes exposed to dsRNA targeting cyp-35d1 compared with gfp controls across the 6 h time course (Figure 2c). This metabolite had a mass of 380.09 Da and was also detected in culture medium of gfp controls after 6 h of TBZ exposure. This metabolite was putatively identified as a glucose conjugate of TBZ-OH since the same fragment ions for TBZ-OH are also revealed during fragmentation (Supplementary Figure S2). Interestingly, peaks consistent with sulfate or glucuronide conjugates of TBZ-OH (as found in mammals) were not identified.

Excretion of TBZ-O-glucose metabolite may be mediated by more than one PGP

Since exposure to the PGP inhibitor verapamil eliminated all differences between susceptible and resistant isolates of the cattle-parasitic nematodes Cooperia oncophora and Ostertagia ostertagi [34], we screened (single and dual) pgp deletion mutants under TBZ exposure to investigate if any of the 15 pgps found in C. elegans are responsible for excretion of the TBZ-O-glucose metabolite identified in the present study. Increased susceptibility to TBZ was found in the pgp deletion strains NL132 pgp-1 (pk17) IV and NL131 pgp-3 (pk18) X. In the separate absence of pgp-1 and pgp-3 nematodes were approximately twice as susceptible to TBZ (P < 0.005; Figure 3), with an LD50 of ~0.2 mM, compared with > 0.4 mM for N2 wild-type.

cyp-35d1 mediated metabolism affects susceptibility to TBZ in wild-type and resistant backgrounds

To investigate the effect of knocking down transcript for cyp-35d1 in the absence of the β-tubulin BEN-1 both nhr-176 and cyp-35d1 were individually targeted by RNAi during TBZ exposure in the CB3474 ben-1 (e1880)III deletion mutant and the number of progeny was counted after 3 days. Half maximal effective concentrations (EC50) for TBZ were estimated for N2 wild-type and the ben-1 deletion mutant when fed on dsRNA targeting gfp, cyp-35d1 and nhr-176. Knockdown of nhr-176 and cyp-35d1 makes N2 wild-type approximately twice as susceptible

Figure 1 Normalized expression of cyp-35a3, cyp-35a5, cyp-35c1 and cyp-35d1 relative to that of ama-1 during a 6 h exposure to DMSO and TBZ when gfp, nhr-176 and cyp-35d1 were targeted by dsRNA in ~10⁴ adults

Under exposure to DMSO expression of cyp-35a3, cyp-35a5, cyp-35c1 and cyp-35d1 was < 1.0, < 1.5, < 3.0 and < 0.1 respectively. Expression of cyp-35a3, cyp-35a5 and cyp-35c1 were induced 76 ± 23.8-, 46 ± 10- and 20 ± 9-fold, whereas that of cyp-35d1 was induced 2019 ± 280-fold in gfp controls after 1 h TBZ exposure. Results are means ± S.E.M. (n ≥ 3).
Figure 2  Relative abundance of (a) TBZ (b) TBZ-OH and (c) TBZ-O-glucose during a 6 h exposure to TBZ when gfp and cyp-35d1 were targeted by dsRNA in ∼10^4 adults

Results are means ± S.E.M. (n ≥ 3); mAu, milli-arbitrary units.

DISCUSSION

Characterization of the pathways by which anthelmintic drugs are modified, metabolized and excreted in nematodes is fundamental to understanding their efficacy, spectrum of activity and mechanisms of resistance. Although metabolism of drugs has been extensively studied in mammals, the extent to which drugs are metabolized in nematodes remains to be determined [9,35,36]. Consequently we have investigated the metabolic pathway of TBZ in C. elegans and its regulation. We have shown previously that cyp-35d1 mediates TBZ metabolism in C. elegans and that induction of this gene is regulated by nhr-176 [9]. In the present study we investigated the ability of recombinant NHR-176 to directly bind TBZ in vitro. We also assessed the effect of knocking down transcripts for both cyp-35d1 and its transcription factor...
nhr-176 on other TBZ-inducible CYP genes and on metabolite production. The potential involvement of PGPs in the excretion of TBZ metabolites was also investigated in deletion mutants. The effect of targeting both cyp-35d1 and nhr-176 in the absence of the TBZ β-tubulin target BEN-1 was also determined.

In mammals, NHRs (PXR/CAR) bind to xenobiotics and activate expression of metabolic enzymes. Since NHR-176 regulates cyp-35d1 under TBZ exposure in C. elegans, we used ITC to investigate whether or not NHR-176 directly binds TBZ. We were able to show that a recombinant form of NHR-176 binds to TBZ under physiologically relevant conditions. Mammalian NHRs have been shown to bind a wide range of endogenous and xenobiotic compounds to regulate metabolic genes [37]. For example, PXR in mammals has a relatively large and flexible ligand-binding site and is able to bind a wide range of ligands [15]. It has also been suggested that other NHRs participate with PXR and CAR in regulating xenobiotic metabolizing enzymes in humans [38]. Peroxisome-proliferator-activated receptors (PPARs) regulate members of CYP1A, CYP2A, CYP2B, CYP2C, CYP2E, CYP2J and CYP3A subfamilies as well as some conjugating enzymes [e.g., EPHX2 (epoxide hydrolase 2), GSTA and UGT1A9; 39]. Specific xenobiotic bio-transformation pathways do not appear to be highly conserved and differences have been found even within mammals. For example, PPARα down-regulates some phase I metabolism genes in mice, including CYP2C11, CYP2C12 and CYP2C29 [40]. NHR-176 belongs to the supplementary group of NHRs which have undergone extensive diversification in nematodes from a common steroid receptor, HNF4 (hepatocyte nuclear factor 4) [41]. The ligand-binding specificity of NHR-176 remains to be tested but it is likely that NHR-176 binds other lipophilic ligands which are abundant in the soil.

Q-PCR analysis shows that cycd-35d1 expression is highly inducible by TBZ and confirms previous expression analysis in GFP reporters [9]. Furthermore, we show that induction of cyp-35d1 by TBZ is prevented by RNAi knockdown of nhr-176 to the same extent as by RNAi knockdown of cyp-35d1 itself (17 ± 3 % and 15 ± 4; Figure 1). The expression of NHR-176 is not affected by TBZ, suggesting that induction of cyp-35d1 is not the result of a change in the expression level of NHR-176 per se. We conclude that when TBZ binds to NHR-176, this activates expression of cyp-35d1. Interestingly, other TBZ-inducible CYP genes (cyp-35a3, cyp-35a5 and cyp-35-c) showed a significantly stronger induction during RNAi knockdown of nhr-176 and cyp-35d1 than in the gfp control (P < 0.05 and P < 0.01; Figure 1), particularly after 6 h of TBZ exposure. Furthermore, microarray and GFP reporter analyses show the expression of both cyp-35c1 and cyp-35a5 is strongly induced under ABZ (albendazole) exposure [36] and may play an important role in oxidation of this BZ. This suggests compensatory regulation by other transcription factors during knockdown of both genes, which may allow some hydroxylation of TBZ to occur. Induction of cyp-35c1 by β-naphthoflavone and fluoranthene is partially regulated by the Mediator subunit MDT-15 [42]. MDT-15 also interacts with NHR-49 to regulate expression of fat metabolism genes and is able to bind several other supplementary NHRs which have undergone extensive diversification in nematodes [41,43]. Since a similar rapid expansion has also occurred within the CYP superfamily in C. elegans [44], it is likely that other NHRs play an important role in regulating oxidation of various xenobiotic ligands as suggested previously [45].

The functional significance of TBZ induction of cyp-35d1, we observe, indicates that this enzyme could catalyse the metabolism of TBZ. To test this, we measured the abundance of TBZ and its putative metabolites (those known to be produced in mammals) in nematodes exposed to RNAi targeting cyp-35d1 and gfp controls prior to TBZ induction. The steady accumulation of TBZ-OH and its persistence through the rest of the time course suggests that the CYP-35D1 protein is longer-lived than the transcript. HPLC–UV analysis suggests that TBZ was metabolized more rapidly in the RNAi controls than when cyp-35d1 was knocked down and confirms that cyp-35d1 catalyses the hydroxylation of TBZ (Figures 2a and 2b). Furthermore, the abundance of a metabolite which was putatively identified as a glucose conjugate of TBZ-OH was also significantly lower in nematodes exposed to dsRNA targeting cyp-35d1 compared with gfp controls across the 6 h time course (Figure 2c). Formation of this metabolite is therefore dependent on prior hydroxylation by CYP-35D1. This hydrophilic compound is likely to be rapidly excreted and was also detected in culture medium after 6 h of TBZ exposure in gfp controls (result not shown). Glucosidation is not a common pathway in mammals and has not been reported in any studies which have investigated metabolism of TBZ or ABZ in mammals [10,11,46–49]. Interestingly, glucose conjugates of ABZ have also been reported in C. elegans and the parasitic nematode Haemonchus contortus which suggests that bio-transformation pathways could be conserved in strongylid nematodes [36,50]. Given that these nematodes include many human and veterinary parasites, revealing the components of these pathways could have important implications both for drug potency and for potential mechanisms of drug resistance.

The pathway by which this TBZ-O-glucose metabolite is formed in C. elegans is yet to be determined. It is possible that ugt-8 could be responsible for glucose conjugation of the TBZ metabolite in C. elegans as this gene is strongly expressed in the intestine under TBZ exposure [9]. Furthermore, the potential for a human UGT to conjugate glucose (as well as glucuronate) to xenobiotics has been demonstrated [51–53]. The regulation of ugt-8 under TBZ exposure is not controlled by nhr-176 in C. elegans and is yet to be discovered [9]. Screening of deletion strains under TBZ exposure for all 15 pgp genes identified two pgp genes in the present study which could be responsible for the excretion of the TBZ-O-glucose metabolite. Increased susceptibility to TBZ was found in deletion strains NL132 pgp-1 (pk17) 1V and NL131 pgp-3(pk18) X (P < 0.005; Figure 3). A higher susceptibility to ivermectin has also been reported in both of these strains [54] as well as increased sensitivity to chloroquine and colchicine in NL131 pgp-3(pk18) X [40]. Consistent with the expression of cyp-35d1, the expression of pgp-1 and pgp-3 are localized to the adult intestine and/or excretory cells [55–58]. An increase in PGP expression has been found in ivermectin-resistant strains of C. elegans [59] and also H. contortus [60]. A number of mutations in pgp genes have also been observed between ivermectin/TBZ-resistant and -susceptible isolates of H. contortus [61-64]. Furthermore, exposure to the PGP inhibitor verapamil eliminated all differences between susceptible and resistant isolates of the cattle-parasitic nematodes C. oncophora and O. ostertagi [34]. Given the wide repertoire of PGPs in C. elegans, it is possible that multiple genes could be operating in combination during excretion of a single metabolite as suggested previously [65].

Since metabolism can limit the efficacy of drugs or pesticides in the whole organism, we compared the potency of TBZ under RNAi targeting cyp-35d1 and nhr-176 to gfp (controls). In the present study we show that knockdown of cyp-35d1 and its transcription factor makes wild-type nematodes and resistant, CB3474 ben-1(e1880) III deletion mutants at least twice as sensitive to TBZ as gfp controls (P < 0.005 and P < 0.001; Figure 4). That TBZ retains some potency in the absence of the β-tubulin BEN-1 suggests that TBZ has additional targets in
C. elegans, which may be other β-tubulins or unrelated proteins. TBZ has been suggested previously to inhibit activity of the helminth-specific fumarate reductase and succinate dehydrogenase in Trichinella spiralis and Strongyloides ratti [66,67]. More recently, TBZ has also been shown to act as a vascular disrupting agent in vertebrates by inhibiting the ρ kinase pathway [68]. The ben-1 deletion mutant and wild-type N2 were relatively resistant to TBZ-OH and knockdown of nhr-176 did not alter resistance (result not shown). This suggests that TBZ itself contributes to more toxicity than the hydrolysed form, which is rapidly excreted in C. elegans. Furthermore, TBZ itself has been shown to directly bind H. contortus tubulin [69]. Our study demonstrates that the potency of TBZ is a synthetic effect of at least three drug–protein interactions: with BEN-1, NHR-176 and CYP35D1 (with interactions with other CYPs and other targets implied but as yet uncharacterized). This highlights the complexity of drug potency, a complexity that is rarely assayed in drug discovery and optimization programmes. By discovering new drug–protein interactions, studies such as ours therefore enable future drug discovery.

Conclusion

We have shown that NHR-176 directly binds TBZ at physiologically relevant concentrations. We have used RNAi in conjunction with HPLC–UV and Q-PCR to show that CYP-35D1 catalyses hydroxylation of TBZ. We have also identified a further metabolite of TBZ-OH (a glucose conjugate) and shown that production of both metabolites is dependent on cyp-35d1. This bio-transformation pathway appears to be unique to nematodes and may have important implications for their effective chemotherapeutic control. We further show that TBZ is likely to have further binding sites to the known BEN-1, β-tubulin target in C. elegans, since knockdown of cyp-35d1 and its transcription factor make nematodes more susceptible to TBZ under wild-type and ben-1 deletion backgrounds. This provides unique evidence that a transcription factor can directly bind an anthelmintic to activate expression of a metabolic pathway enhancing its solubility and excretion.

AUTHOR CONTRIBUTION

Laura Jones, Anthony Flemming and Peter Urwin conceived and designed the experiments. Laura Jones performed the experiments and analysed the data. Laura Jones, Anthony Flemming and Peter Urwin drafted the paper.

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C. elegans wild-type Bristol N2 strain, CB3474 ben-1(e1880) III and pgp deletion strains NL132 pgg-1(pkol17) V, GH378 pgg-2(ok484) I, NL131 pgg-3(pkol18) X, VC2158 pgg-4(pkol106) X, RB859 pgg-5(ok856) X, RB1047 deletion in pgg-7 and pgg-6(pkol894) X, RB1916 pgg-8(ok2489) X, RB1045 pgg-10(ok991) X, VC262 pgg-12(ok19) X, RB894 pgg-13(ok1477) X, RB2008 pgg-14(ok2660) X and RB1041 pgg-15(ok897) X were obtained from the Caenorhabditis Genetics Center (CGC) at the University of Minnesota, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440). Mutant strains for pgg-9 (tm0830) and pgg-11 (tm3033) were provided by the National Bioresource Project, MlTANI Laboratory at Tokyo Women’s Medical University. MS analysis was carried out by Dr James Ault and technical advice was given by Dr lain Mannick for HPLC.

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