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CHARACTERIZATION OF THE XENOBIOTIC RESPONSE OF *CAENORHABDITIS ELEGANS* TO THE ANTHELMINTIC DRUG ALBENDAZOLE AND THE IDENTIFICATION OF NOVEL DRUG GLUCOSIDE METABOLITES

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Synopsis

Knowledge of how anthelmintics are metabolized and excreted in nematodes is an integral part of understanding the factors that determine their potency, spectrum of activity and of investigating mechanisms of resistance. Although there is remarkably little information on these processes in nematodes, it is often suggested that they are of minimal importance for the major anthelmintic drugs. Consequently, we have investigated how the model nematode *Caenorhabditis elegans* responds to and metabolizes albendazole; one of the most important anthelmintic drugs for human and animal use. Using a mutant strain lacking the β -tubulin drug target to minimize generalized stress responses, we show the transcriptional response is dominated by genes encoding xenobiotic metabolizing enzymes (XME), particularly cytochrome P450s and UDP-glucuronosyl transferases (UGTs). The most highly induced genes are predominantly expressed in the worm intestine, supporting their role in drug metabolism. HPLC-MS/MS revealed the production of two novel glucoside metabolites in *C. elegans* identifying a major difference in the biotransformation of this drug between nematodes and mammals. This is the first demonstration of metabolism of a therapeutic anthelmintic in *C. elegans* and provides a framework for its use to functionally investigate nematode anthelmintic metabolism.

Short (page heading) title: Induction of XME and glucosidation of ABZ in *C. elegans*

Keywords: Nematode, anthelmintic resistance, metabolism, cytochrome P450, UDP-glucuronosyl transferase

Abbreviations footnote: The abbreviations used are: ABZ, albendazole; ABZ-SO, albendazole sulphoxide; ABZ-SO₂, albendazole sulphone; FDR, false discovery rate; GST, glutathione-s-transferase; MeCN, acetonitrile; m/z, mass/ charge ratio; PPAR, peroxisome proliferator- activated receptor; RP, rank products; UGT, UDP-glucuronosyl transferase; XME, xenobiotic metabolizing enzyme

Introduction

Resistance of parasitic nematodes to the commonly used anthelmintic drugs is a major problem in livestock and an emerging problem in humans [1-4]. Consequently, there is an urgent need to understand the mechanisms of anthelmintic resistance, to develop approaches that optimize drug potency and to develop new synergists and drugs. An understanding of the way in which anthelmintic drugs are modified, metabolised and excreted from nematodes is central to these questions. Metabolism of pesticides in insects is known to have a major effect on drug potency and has been identified as playing a major role in insecticide resistance in a number of different insect species [5-7]. However, there has been remarkably little work in this area for nematodes and it has commonly been suggested that oxidative metabolism of xenobiotics is unimportant in this class of organism [8-10]. This has led to these processes

being ignored as mechanisms of anthelmintic resistance or as important factors to consider in anthelmintic drug design and modification. However, examination of the genomes of free-living and parasitic nematodes reveals the presence all the key enzyme families that are known to be involved in these processes in vertebrates and insects. Recent work has shown some members of these families, most notably the cytochrome P450s are inducible on exposure to a range of organic small molecules [11-13]. In addition Kulas *et al.* (2008), demonstrated a discreet 450nm absorption peak in carbon monoxide bound, dithionate treated *C. elegans* microsomes preparations, supporting the presence of active cytochrome P450 protein in the nematode [14]. However, surprisingly, nematode xenobiotic responses to and biotransformation of any of the therapeutically important anthelmintic drugs have yet to be investigated.

Albendazole belongs to the benzimidazole (BZ) class of anthelmintics and is one of the most important anthelmintics used to control parasitic nematodes in both humans and animals. This class of drug acts by binding to β -tubulins and disrupting microtubule formation and mutations in the *ben-1* β -tubulin gene result in high levels of resistance in *C. elegans* [15]. Mutations of homologous tubulin genes in parasites (the isotype-1 β -tubulin genes) have since been associated with BZ resistance in many parasitic nematode species including *Haemonchus contortus*, *Cooperia oncophora*, *Teladorsagia circumcincta* and *Trichostrongylus colubriformis* [16-20]. Although mutations in β -tubulin are clearly important, they are only part of the story. It is notable that *H. contortus* populations homozygous for the β -tubulin mutations often have markedly different levels of resistance [21]. Hence, there are clearly additional important mechanisms of resistance for the benzimidazole drugs and it is increasingly suggested that other mechanisms may be involved [22,23]. In the case of triclabendazole resistance in *Fasciola hepatica*, recent studies suggest that metabolism of the drug to an inactive form by the fluke may be a mechanism of resistance [24].

Pharmacokinetic analyses have shown that albendazole, and the other benzimidazoles, are highly susceptible to biotransformation in mammals [25,26]. Albendazole is almost entirely converted to the active metabolite albendazole sulphoxide (ABZ-SO) during first-pass metabolism. This reaction is thought to be primarily catalysed by flavin monooxygenase and the CYP3A family [27-29]. Further sulphoxidation to the inactive albendazole sulphone (ABZ-SO₂) is thought to occur via the CYP1A family [27]. Like many xenobiotics, the presence of albendazole and its metabolites has been shown to induce cytochrome P450 enzymes and other xenobiotic metabolising enzymes in many species [29-32]. The nature of xenobiotic responses to anthelmintics and the extent to which these drugs are metabolized in nematodes is unknown and has been subject to conflicting reports [33-37]. This is an important question both with regard to potential mechanisms of anthelmintic resistance in parasites and to determining possible limiting factors for potency and spectrum of current drugs and novel lead compounds. Consequently, we are developing *C. elegans* both as a model system in which to investigate nematode drug metabolism and as a heterologous system for the functional analysis of relevant parasite genes. We have chosen to first investigate albendazole because it is a therapeutically important member of the benzimidazole anthelmintic class which is known to be a good substrate for biotransformation enzymes in mammals.

We have investigated the transcriptional response of *C. elegans* to albendazole exposure utilizing a drug resistant mutant to minimize responses associated with non-specific stress or with the drug's mode of action. We have found that that under these conditions, genes encoding drug metabolizing enzymes, particularly cytochrome P450s and UDP-glucuronosyl transferases, dominate the transcriptional response. We have also demonstrated significant metabolism of albendazole in *C. elegans*, which results in the production of novel glucoside metabolites. Glucosidation of xenobiotics is extremely rare in mammals and there are no reports of albendazole glucosidation in mammalian systems. This may represent an important difference between mammalian and nematode xenobiotic responses in general and could potentially be a target for future chemotherapeutics. This work demonstrates that the benzimidazole drug albendazole is a potent inducer of nematode drug metabolizing enzymes and a substrate for their action and establishes *C. elegans* as an appropriate system in which to functionally investigate these processes.

Experimental Procedures

C. elegans strains- In order to minimize the transcriptomic changes caused by stress secondary to the effect of the drug, the benzimidazole (BZ) resistant strain, CB3474, was used in all drug exposure experiments. CB3474 worms contain the mutation *ben-1(e1880)III* [15], which is dominant at 25°C and recessive at 15°C. The wild-type strain used for expression pattern analysis was Bristol N2 a DR subclone of CB original (Tc1 pattern I). Both strains were gifts from the *C. elegans* Genetics Centre (CGC). Worms were maintained at 15-20°C on NGM plates with an OP50 bacterial lawn.

Synchronization of cultures- Embryos were isolated by hypochlorite treatment of gravid adults [38]. The embryos were transferred to a 5 cm diameter Petri dish in 6 ml of S-buffer (129 ml/L 0.05M K₂HPO₄, 871 ml/L 0.05M KH₂PO₄, 0.1M NaCl; pH 6.0), and maintained at 20°C overnight to allow them to hatch. The concentration of L1 larvae was assessed the following day and the larvae were used to start experimental cultures the same day.

Anthelmintic exposures- Standard liquid culture methods [38] were used with the exception that water soluble cholesterol-polyethylene glycol 600 sebacate (Cholesterol-PEG; Sigma, UK) was used at a final concentration of 25 µg/ml. This appeared to increase the solubility of the drug compared to the use of standard cholesterol. Approximately 10000 L1 larvae were added to each of two 50 ml cultures, containing 1ml concentrated OP50. The worms were grown at 20°C, with shaking at 240 rpm for 70 hrs, until many young adults were present. 450 µl of 20 mg/ml albendazole (Sigma) in DMSO was added to one flask (final concentration 300 µg/ml ABZ) and 450 µl of DMSO to the other (final concentration of DMSO 1.5% v/v). The cultures were grown for a further 4 hrs, harvested by sucrose flotation [39] and snap frozen in liquid nitrogen until RNA was extracted.

Separate biological replicates were carried out for analysis by real-time QPCR. These were carried out in an identical manner except for the use of a commercial preparation of albendazole (Albex 10%, Chanelle). Investigation of gene up-regulation following exposure to a gradient of albendazole concentrations was also undertaken. Five matched cultures of *C. elegans* were prepared. Cultures were exposed to 0.3, 3, 30, 300 µg/ml or no ABZ control for 4 hrs. Albendazole (Sigma) dissolved in DMSO (stock 20 mg/ml) was used and all cultures contained 1.5% v/v DMSO.

RNA methods- RNA was extracted using Trizol (Invitrogen) reagent according to the manufacturers guidelines. Briefly, pelleted worms were homogenised in four volumes Trizol reagent, subject to two chloroform extractions and precipitated in isopropanol. The RNA was then treated with RNase-free DNase I (Qiagen) in solution before purification and concentration using RNeasy columns (Qiagen). Quality and concentration of RNA was assessed using an Agilent Bioanalyser 2100. Total RNA destined for microarray analysis was precipitated in ethanol until analysis. RNA for real-time QPCR analysis was reverse transcribed using a cloned AMV first strand synthesis kit (Invitrogen) and random hexamer primers. 5 µg total RNA for each sample was used as template and an identical reaction lacking reverse transcriptase enzyme was carried out simultaneously. cDNA was purified using PCR purification columns (Qiagen), resuspended in 30 µl TE buffer and stored at -80°C until use.

Microarray hybridization and analysis- Sample labeling and hybridization to *C. elegans* whole genome Genechips (Affymetrix) were performed using standard Affymetrix protocols (http://media.affymetrix.com/support/downloads/manuals/expression_analysis_technical_manual.pdf). These chips contain oligonucleotide probesets designed to assess over 22500 transcripts from the *C. elegans* genome. An updated annotation dataset was assembled for the *C. elegans* probesets present on the Genechip. Data were sourced from WormBase (Sept. 2008). Scanned array images (CEL files) were quality control assessed using the arrayQualityMetrics Bioconductor package (www.bioconductor.org) in the R environment (www.r-project.org). Arrays identified as possible outliers were removed from subsequent analyses. Linear model fitting of the array data was undertaken, taking into account biological replicates using the limma (Linear Models for Microarray Data) Bioconductor package (www.bioconductor.org/packages/bioc/html/limma.html). The Rank Products algorithm was used to assess differential expression of genes between test and control groups and to assign significance to these

changes [40]. Assignment of significance was carried out using a False Discovery Rate (FDR) cut-off of 5-10%.

Gene Ontology analysis- DAVID software (the Database for Annotation, Visualisation and Integrated Discovery) from the National Institutes of Health was used to assess the functional annotation and clustering of the genes noted to be differentially expressed between samples [41,42]. Input into the program consisted of probesets shown to be significantly altered in expression using the Rank Products algorithm, with a false discovery rate (FDR) of less than 10%. Prevalence of annotation terms within the list of differentially expressed genes were compared to the prevalence in the whole *C. elegans* genome. Fold enrichment was calculated and a modified Fishers exact test (EASE score) used to assign significance. Gene functional classification clustering was carried out using medium stringency.

Real-time Quantitative PCR- Relative quantitation of genes of interest was assessed using Brilliant SYBR Green QPCR master mix (Stratagene) and a Stratagene Mx 300P QPCR system with Stratagene MxPro software. *ama-1*, encoding a subunit of RNA polymerase II, was used as a normalising gene. This constitutively expressed gene showed no significant changes on microarray analysis and has been extensively used as a normalising gene in differential expression studies in *C. elegans* [44]. Gene specific primers were designed to produce a product between 160 and 200 bp in length. The sequence of these primers can be found in **Supplementary Table 1**. The final concentration of primers was between 300 and 400nM in a total reaction volume of 25 μ l.

Expression pattern analysis- GFP reporter constructs for *cyp-35C1*, *cyp-35A5*, *cyp-35A2*, *ugt-63*, *ugt-16*, *gst-5*, C29F7.2 and T16G1.6 were created using a PCR fusion protocol as described by Hobert *et al.* [44]. The putative promoter region, 3Kb upstream from the ATG start site of the gene of interest, was fused to the *gfp* gene, including synthetic introns and *unc-54* 3' UTR, from Fire vector pPD95.67 [45]. In addition the promoter region of one gene, *cyp-35C1*, was cloned into the GFP reporter plasmid PJM-355*. Both of the plasmids used contained a *gfp* gene with a nuclear localization signal. Transgenic lines were created using the method of Mello *et al.* [46], with the plasmid pRF-4 as a co-transformation marker to identify transgenic worms. Expression patterns were visualized using a Zeiss, Axioscop 2 plus microscope. Images were collected and processed using Improvion Openlab software (www.improvision.com).

HPLC-MS- Paired 250 ml liquid cultures containing many mixed stage CB3474 worms were grown over 4-5 days at 20°C, 240 rpm. One of the paired cultures was killed by heating to 50°C for 30 min in a waterbath. Albendazole was added to both the experimental and heat-killed culture to a final concentration of 15 μ g/ml. Following 7hrs culture with ABZ the worms were harvested by centrifugation and washed three times in M9 buffer. Packed nematodes were homogenized on ice using both an Ultra Turrax T8 homogenizer (IKA-Werke) and sonication with a Soniprep 150 (Sanyo). ABZ and metabolites were extracted in ten volumes of methanol: Tris pH9, 9:1. The samples were centrifuged at 4000rpm, 4°C for 40 min to remove solid debris and the supernatant evaporated to dryness under nitrogen at 40°C using a Turbovap LV (Zymark). The residue was resuspended in 200 μ l of 50:50 acetonitrile: deionized milli Q (MQ) water supplemented with 0.1% formic acid and centrifuged at 10000 rpm to remove solids. Bacterial controls were carried out in a similar manner using cultures containing no nematodes and 3ml concentrated OP50 suspension per 250ml culture.

5 μ l of resuspended residue was injected onto a Waters HSS 1.8 μ m C18 column (100 x 1mm), using a Waters Acquity Ultra Performance LC system. Mobile phase, a gradient of 95:5% water: MeCN + 0.1% Formic acid to 100% MeCN + 0.1% FA over 12min, was applied to the column at 200 μ l/min. Tandem mass-spectrometry analysis was carried out using a Micromass MS Technologies Q-ToF PremierTM. A reference spray using leucine enkephalin was used to provide accurate mass data. A standard solution of albendazole was analysed alongside experimental samples and mass readings were correct to within +/- 16 ppm (0.004 Da). Analysis was carried out by Q1 and MS/MS methods using Mass Lynx 4.1 software (Waters).

Results

Characterisation of the transcriptional response to albendazole using whole genome microarrays. Three biological replicates of *C. elegans* exposed to 300 µg/ml of albendazole for 4 hours were prepared along with paired negative controls. Total RNA was extracted and hybridised to Affymetrix *C. elegans* arrays. The majority of genes show no change in expression level (**Figure 1A**). Analysis of the data using the rank products algorithm showed 33 probesets to be significantly up-regulated and three probesets to be significantly down-regulated with a false discovery rate of 5%. Further analysis focussed on the up-regulated genes, as these were more likely to encode enzymes involved in xenobiotic metabolism. However, full microarray data can be found in **Supplementary Table 2** and has been uploaded to the GEO repository (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE21747>).

In order to broaden the global analysis, up-regulated genes with an FDR <10 % were subject to analysis using DAVID (Database for Annotation, Visualisation and Integrated Discovery) software. This data set contained 51 probe sets, which represented 42 genes in the *C. elegans* genome. There was enrichment of several gene ontology terms associated with transferase and monooxygenase enzymes (**Figure 2**). These classes of enzyme are common within xenobiotic metabolism pathways. 20 genes from the list had no gene ontology terms associated with them. Therefore, to increase the coverage of annotation, protein domain terms, functional category terms and KEGG pathway terms were assessed in addition to gene ontology terms. Using this method only six genes were not annotated, all of which were uncharacterised hypothetical proteins. Clustering of the up-regulated genes based on annotation term co-occurrence revealed there to be two gene families up-regulated. Cluster 1, enrichment score 8.66, represents eight genes which are confirmed or putative members of the UDP-glucuronosyl transferase family (**Figure 2**). Cluster 2, enrichment score 2.64, represents five genes, four of which are members of the cytochrome P450 family (**Figure 2**). The fifth gene in cluster 2 is *vem-1*, which represents a cytochrome b5-like transmembrane protein. Cytochrome b5 has been shown by many groups to interact with many cytochrome P450 reactions where it is thought to be important as an electron donor, reviewed by Schenkman and Jansson [47]. Additionally, *vem-1* has been reported to be induced in response to exposure to xenobiotics such as β-naphthoflavone and clofibrate [12].

Several of the up-regulated genes that were not clustered encode potential xenobiotic metabolism enzymes including an alcohol dehydrogenase and a glutathione-s-transferase (**Supplementary Table 2**). Other up-regulated genes include predicted small molecule kinases and *jnk-1*, another kinase likely involved in signalling cascades, and the metallothionein encoding gene *mtl-1*, which we have also found to be up-regulated in response to ivermectin exposure (Laing, Ivens and Gilleard, unpublished data). Many of the other up-regulated genes have unknown function. However, ten of these genes, all containing putative CUB domains (for complement C1r/C1s, Uegf, Bmp1), have been shown to be coincidentally up-regulated with *cyp-35A5*, *gst-5* and *vem-1* in response to infection with the toxin secreting bacterium *Pseudomonas aeruginosa*, **Supplementary Table 2** [48].

The ten probesets showing the greatest up-regulation represent a putative xenobiotic metabolism pathway. The top 10 gene list, based on log₂ fold change, includes three *cyps*, two *ugts* and a *gst* (**Figure 1B**). Of the three probesets not representing genes encoding xenobiotic metabolising proteins only one is completely uncharacterised. The other two both represent predicted small molecule kinases, which could potentially be involved in the signalling cascade in response to albendazole. Up-regulation of all of these genes in response to albendazole exposure was confirmed using real-time QPCR (**Figure 3A**). The fold change of specific genes was higher using RT-QPCR than that suggested by microarray experiments, as has been noted in several other studies [49,50]. Two control genes, which showed no change in expression in the microarray experiments, were included in the RT-QPCR analysis, in addition to the normalising gene *ama-1*. The lack of any alteration in expression level of *col-19*, an adult specific collagen gene, not only serves to confirm the accurate staging of the control and drug exposed populations but acts as a negative control for the RT-QPCR technique. Similarly *cyp-37B1*, which we have found to be up-regulated in response to ivermectin exposure (Laing, Ivens and Gilleard, unpublished

data), was not significantly up-regulated following ABZ exposure using microarray analysis or RT-QPCR.

cyp-35C1, cyp-35A5, cyp-35A2 and ugt-16 respond to ABZ in concentration dependent manner. RT-QPCR was used to assess the fold up-regulation of *cyp-35C1, cyp-35A2, cyp-35A5* and *ugt-16* following exposure to a range of ABZ concentrations for 4 hrs (**Figure 3B**). Whilst *cyp-35A5* showed a 2-3 fold change at 0.3 µg/ml ABZ, the other three genes investigated did not show any convincing up-regulation. Modest up-regulation of all genes examined was evident following 4 hours exposure to 3 µg/ml, but maximal fold changes were noted at concentrations of 30 µg/ml ABZ or greater.

ABZ-responsive genes are expressed in the intestine. GFP reporter gene fusions were constructed for eight of the genes showing the greatest up-regulation following exposure to ABZ. The promoter regions of *cyp-35C1, cyp-35A5, cyp-35A2, C29F7.2, T16G1.6, gst-5* and *ugt-16* drove GFP expression predominantly in the intestine (**Figure 4**). This organ has been proposed as the main site of detoxification in *C. elegans* [51]. *gfp* expression under the control of the *ugt-63* promoter was localised to the hypodermis.

Demonstration of albendazole metabolism in C. elegans and the production of novel glucoside metabolites. Organically extracted homogenates of *C. elegans* following exposure to albendazole for 7 hrs were initially analyzed using a total ion scan with accurate mass analysis. As well as an intense peak for albendazole at approximately 4.68 minutes there were also significant peaks for albendazole sulphoxide (mass 282.091 Da, elution time 3.18min), two glucose conjugates (mass 428.149 Da, elution times 4.06 and 4.26 min) and a metabolite of mass 208.092 Da, elution time 4.22 min), (**Figure 5**). The metabolite of mass 208.092 Da is consistent with a cleavage across the amino bond of ABZ (loss of COOCH₃) and will be referred to as amino-ABZ. The peak intensities of both the ABZ-SO and amino-ABZ peaks were extremely variable and both metabolites were also found in heat-killed nematode controls and bacteria only controls suggesting they are not products of nematode metabolism. However, the glucose conjugates of albendazole were unique to the experimental live worm samples and their identities were confirmed using MS-MS fragment analysis (**Figures 5 and 6**). Fragmentation of albendazole typically reveals three major fragment ions: 234.06 Da, 191.01 Da and 159.04 Da. Fragmentation of the proposed glucose conjugates of albendazole identified only two major fragments. One of mass 266.09 Da is proposed to be albendazole itself and the other of mass 234.06 Da is likely to be the albendazole acylium, caused by protonation of the carbamate, which is also present in the albendazole fragment ion spectrum. These findings confirm the identity of the peaks of mass 428.149 Da as metabolites of albendazole and the mass change is consistent with them being glucose conjugates. The fragment ion spectra for each of the glucoside metabolites are identical. Therefore, whilst the molecular position at which the glucose has been conjugated is not certain, it is likely that the conjugation sites are the two benzimidazole nitrogens; which are the same number of bonds from the sulphur and the carbamate ester and share the double bond due to resonance. Interestingly, there were no peaks consistent with ABZ-SO₂ (expected mass 298.09) or glucuronidation of albendazole (expected mass 442.09 Da).

The peak intensity of the albendazole-glucoside metabolites were between 0.7-16 % of that of albendazole in the analysed incubations. The group of cultures which were pre-exposed to the PPAR α agonist fenofibrate, a potent inducer of cytochrome P450s, UGTs and several other xenobiotic metabolizing enzymes (XMEs), produced a more intense ABZ-glucoside peak (**Figure 6**). Although accurate quantitation of the metabolites was not possible since an albendazole-glucoside standard was not available, there was a clear increase in metabolite production following fenofibrate exposure.

Discussion

There has been much debate, but little experimentation, regarding the extent to which anthelmintics induce xenobiotic responses and are metabolized in nematodes [8-10]. Consequently, we have investigated these processes for a therapeutically important anthelmintic using a benzimidazole resistant strain of the model nematode *C. elegans*, CB3474 *ben-1(e1880)III*. A panel of genes proposed to be up-regulated in response to stress (*sip-1, HSF-1, gst-1, gst-4, gst-38, hsp 16.1, hsp-16.49* and *hsp-70*) showed

no significant changes in expression on the microarrays, suggesting that non-specific stress responses were not induced by exposure of CB3474 to 300ug/ml of albendazole for 4 hours [52-57]. This, together with the fact that such a small number of genes had significant changes in expression level, suggests the up-regulated genes we have identified represent a specific xenobiotic response to albendazole exposure. Within this subset of genes, only the cytochrome P450 and UDP-glucuronosyltransferase families were significantly enriched. In addition, concentration gradient experiments using real-time PCR suggest that up-regulation of *cyp-35C1*, *cyp-35A5*, *cyp35A2* and *ugt-16* occurs at physiologically significant concentrations of drug: the peak plasma and abomasal fluid concentrations of albendazole sulphoxide in sheep have been reported to be 3.2 and 26.2 µg/ml respectively [25].

Members of the *cyp-35* family, including the three genes showing the greatest increase in expression level following albendazole exposure have previously been reported to be inducible by β-naphthoflavone and atrazine [12]; PCB52, fluoranthene and lansoprazole [11,58]; and ethanol [13]. However, up-regulation of the *cyp-35* family does not appear to be a general response to all xenobiotics as these genes are not up-regulated in response to acrylamide [59], clofibrate or diethylstilbestrol [12]. In addition, no members of the *cyp-35* family were induced following exposure to ivermectin (Laing, S., Ivens, A and Gilleard, J.S, unpublished data). The CYP35 family in *Caenorhabditis elegans* is most closely related to the CYP2 family of humans and other mammals. CYP35C1 has closest homology to *H. sapiens* CYP2B6 (BLASTp E-value: 1.1 e-56, 93.9% length). Both CYP35A5 and CYP35A2 bear closest homology to *H. sapiens* CYP2C8 (BLASTp E-value: 4.2 e-53, 93.9% length and 4.6 e-57, 97% length respectively). Whilst these do not appear to be the major CYP isoforms involved in albendazole metabolism in humans, both CYP2B6 and CYP2C8 are thought to play important roles in drug metabolism and are highly inducible [60-62]. Of the two UGTs represented in the top 10 up-regulated genes following ABZ exposure, the predicted polypeptide encoded by *ugt-63* has closest homology to mammalian UGT1A1 (BLASTp E-value: 1.7 e-27, 95.7% length) and that of *ugt-16* has closest homology to UGT2B7 (BLASTp E-value: 6.3 e-43, 95.5% length), both of which are involved in xenobiotic conjugation. Up-regulation of UGT type 1 activities following exposure to ABZ has been noted in the rat and is thought to speed the biological inactivation of the drug in this species [29,30]. In addition, human UGT2B7 has been proposed to utilize UDP-glucose, in addition to glucuronate, to conjugate hyodeoxycholic acid, barbiturates and an endothelin ET (A) receptor antagonist [63-65]. Whilst these relationships are worth noting, species differences in the affinity of specific classes of XME for substrates are common and attempts to draw conclusions from the action of distantly related enzymes should be treated with caution.

Following incubation with *C. elegans* cultures, albendazole was shown to be metabolized to produce albendazole sulphoxide and two albendazole-glucoside metabolites. However, further investigation of ABZ metabolism using axenic cultures of *C. elegans* will be necessary to clarify whether the nematode directly contributes to the ABZ sulphoxidation. This metabolite, which is the major albendazole biotransformation product in mammals, is known to be pharmacologically active and so its significance in terms of drug potency is questionable. However, the two glucoside metabolites are of more interest. They are definitely the products of *C. elegans* metabolism since their production was absolutely limited to the live worm cultures. Glucosidation is an uncommon metabolism pathway in mammals and has not been reported in any of the studies performed on mammalian albendazole metabolism [25,26,32,66]. Hence the metabolism of albendazole by nematodes differs significantly from that in mammals and involves the production of what could be nematode-specific metabolites. A recent study reported that albendazole sulphoxide and two albendazole glucoside conjugates similar to those we identified here for *C. elegans* are produced in adult *H. contortus* nematodes [67]. The similarity of the metabolites produced by *C. elegans* and the parasitic nematode *H. contortus* suggests the biotransformation pathways may be conserved in the strongyloid nematodes, which includes many important human and animal parasites. This is an exciting result as it suggests the dissection of the albendazole metabolism pathway in *C. elegans* will be of relevance to strongyloid parasitic nematodes. Additionally, *C. elegans* should be a valid system for the heterologous expression of parasite enzymes for functional analysis. Although we do not yet know whether the glucoside metabolites are pharmacologically inactive, it seems likely that such a major modification will have a significant effect on the pharmacodynamics and solubility of the drug. Hence

this biotransformation reaction may have important implications both for drug potency and for potential mechanisms of drug resistance.

Cytochrome P450s are ubiquitous haem-containing monooxygenase enzymes, which are involved in the phase I metabolism of many drugs and xenobiotics in mammals and other species. The UGTs are involved in phase II metabolism; by conjugating glucuronate or glucose to drugs directly, or following functionalization by phase I enzymes, they render drugs more hydrophilic so that they can be excreted from the cell and organism. Hence these are the enzyme families likely to be involved in the catalysis of albendazole biotransformation reactions in *C. elegans*. This is supported by the experiment in which prior exposure of *C. elegans* to the PPAR α agonist, fenofibrate, increased ABZ-glucoside metabolite production (**Figure 6**). Drugs of this class are known to be potent inducers of cytochrome P450 and UGTs [68-70]. The family members we have identified as being the most highly induced in response to albendazole exposure are strong candidates for the key catalytic enzymes involved in albendazole glucoside production. It is generally accepted that substrates will induce the expression of the gene encoding the appropriate XME [71]. Consequently, we are undertaking a series of overexpression and gene knockout experiments to test this hypothesis and identify the key enzymes involved.

One final point of interest is that the nature of the transcriptional response to albendazole may provide some clues as to the mechanism of induction. Many of the genes up-regulated in response to albendazole, including *cyp* and *ugt* genes have been proposed as regulatory targets of MDT-15 (**Supplementary Table 3**). MDT-15 is a subunit of mediator, an evolutionary conserved co-regulator of RNA polymerase II, which is thought to be involved in both constitutive and induced expression of several genes of interest [72]. Taubert *et al.* (2008) investigated the targets of the product of *mdt-15* by using whole genome microarrays to compare the transcriptomes of *mdt-15(RNAi)* worms to a control population. Of the 42 genes up-regulated in response to albendazole exposure, 11 were also significantly down-regulated in *mdt-15(RNAi)* worms, and therefore are considered to be regulatory targets of MDT-15. Comparing fold change alone without statistical analyses reveals that even more of the ABZ responsive genes may be targets of MDT-15 (**Supplementary Table 3**). Conspicuously, eight of the top 10 genes in the ABZ up-regulated microarray are regulated by MDT-15. Of the two that do not fit this pattern K08D8.6 showed no change in the *mdt-15(RNAi)* experiment and *ugt-16* was not represented at all in that experiment. Little is known about the regulation of xenobiotic responsive genes in nematodes, representing another important gap in our knowledge of nematode biology.

In conclusion, our study represents the first genome-wide characterization of the xenobiotic response to a therapeutically important drug in any nematode. We have proven that *C. elegans* is capable of metabolizing the benzimidazole drug albendazole, and does so in a completely novel manner compared to any mammal thus far investigated. In addition, this study provides a platform upon which to investigate the xenobiotic response to other anthelmintics, to functionally investigate the role of parasitic nematode XME in anthelmintic metabolism and to further investigate the role of nuclear hormone receptors in xenobiotic responses in nematodes.

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FIGURE LEGENDS

Fig.1. A. Volcano plot representing all probesets on the *C. elegans* Genechip (Affymetrix). Following exposure to ABZ probesets below the solid line showed significant changes in expression with a FDR < 5%. Those below the dashed line showed significant changes in expression with a FDR < 10%

B. Top 10 up-regulated genes following exposure to albendazole. Note the large number of genes belonging to potential xenobiotic metabolizing enzyme (XME) families.

⁺*cyp-35C1* is represented by two probes on the Genechip and appears twice in the top 10.

Fig.2. Ontology terms associated with genes up-regulated in response to 4hrs exposure of strain CB3474 to 300µg/ml ABZ (many of which are overlapping). The EASE score associated with each ontology term is presented under the term. Columns represent the percentage of up-regulated genes associated with each ontology term (total 42 genes) and the numbers at the end of the column are the absolute number of genes.

Clustering of genes based on annotation term co-occurrence reveals a cluster of UGT and a cluster of P450 encoding genes.

Fig.3. A. Relative quantitation of gene expression changes following 4 hrs exposure to albendazole (+/- SEM). All genes assessed by microarray analysis to be up-regulated are confirmed to be up-regulated by RT-QPCR. In contrast the control genes *col-19* and *cyp-37B1* show no change in expression. B. Response of four genes of interest following 4hrs exposure to a gradient of ABZ concentrations (+/- SEM). All genes analysed showed their maximal fold changes at 30µg/ml ABZ. Modest up-regulation of *cyp-35A5* was apparent at 0.3µg/ml ABZ and of both *cyp-35A5* and *cyp-35C1* at 3µg/ml ABZ.

Fig.4. GFP expression patterns of the ABZ responsive genes.

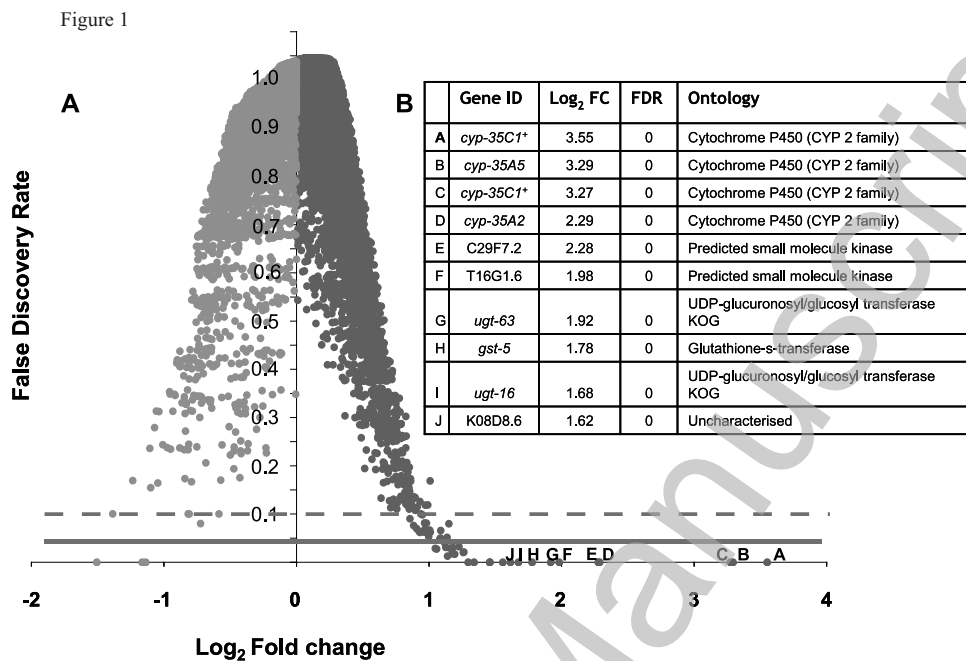
The table shows that all genes thus far examined, with the exception of *ugt-63*, are expressed in the intestine. In all cases expression is seen from L1 to adult stages. Images are included for *cyp-35C1*, *cyp-35A5*, *cyp-35A2*, T16G1.6, *gst-5* and *ugt-16*. GFP fluorescence was weak in strains carrying reporters for C29F7.2 and *ugt-63* and images are not presented.

Fig.5. Chromatograms of albendazole and metabolites from *ex vivo C. elegans* incubation.

Intensity (counts per second) of the peaks of interest is shown in top left corner of each chromatogram and the proposed structures are overlaid. The scanned masses were: ABZ 266.093 Da, ABZ-SO 282.091 Da, amino-ABZ 208.092 Da, ABZ-glucoside 428.149 Da. The *ex vivo* incubations showed intense peaks for ABZ-SO, amino-ABZ and ABZ-glucoside, but peak intensity for all metabolites was significantly lower than that of the parent compound. Comparison of the fragment ion spectra of the proposed ABZ-glucoside metabolites, (b) and (c), and that of ABZ, (a), confirms these peaks as metabolites of albendazole. The exact structures of these metabolites remain unknown, but the nitrogen atoms that are the likely sites of glucose conjugation are highlighted.

* A peak at approximately 9.45 min with the same mass as ABZ-SO (282.088) present in *ex vivo* incubations. MS-MS studies confirmed this was not a metabolite of albendazole.

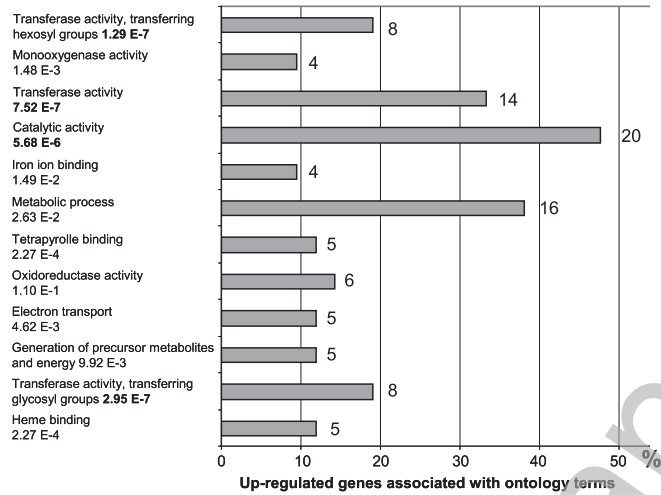
Fig.6. Relative intensity of ABZ-glucoside metabolite (elution time 4.06 min) from cultures with and without pre-exposure to fenofibrate (+/- SEM). Albendazole glucoside production is greater following pre exposure of worm cultures to 20 µg/ml fenofibrate. Note the lack of an albendazole-glucoside peak in the bacterial control group. Graph represents the result of three biological replicates for each condition.



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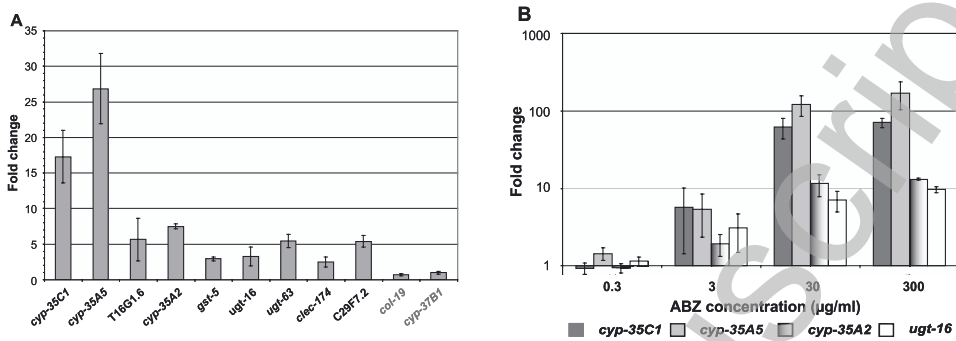
Figure 2



Functional classification group 1		Functional classification group 2	
ID	Description	ID	Description
<i>ugt-1</i>	UDP-glucuronosyl/ glucosyl transferase KOG	<i>cyp-35A2</i>	cytochrome P450
<i>Y43D4A.2</i>	UDP-glucuronosyl/ glucosyl transferase protein domain	<i>cyp-35C1</i>	cytochrome P450
<i>ugt-5</i>	UDP-glucuronosyl/ glucosyl transferase KOG	<i>cyp-35A5</i>	cytochrome P450
<i>ugt-16</i>	UDP-glucuronosyl/ glucosyl transferase KOG	<i>cyp-29A2</i>	cytochrome P450
<i>ugt-25</i>	UDP-glucuronosyl/ glucosyl transferase KOG	<i>vem-1</i>	Putative steroid membrane receptor KOG
<i>ugt-41</i>	UDP-glucuronosyl/ glucosyl transferase KOG		
<i>ugt-63</i>	UDP-glucuronosyl/ glucosyl transferase KOG		
<i>ugt-22</i>	UDP-glucuronosyl/ glucosyl transferase KOG		
<i>ugt-1</i>	UDP-glucuronosyl/ glucosyl transferase KOG		

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Figure 3



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Figure 4

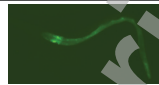


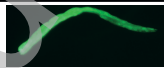


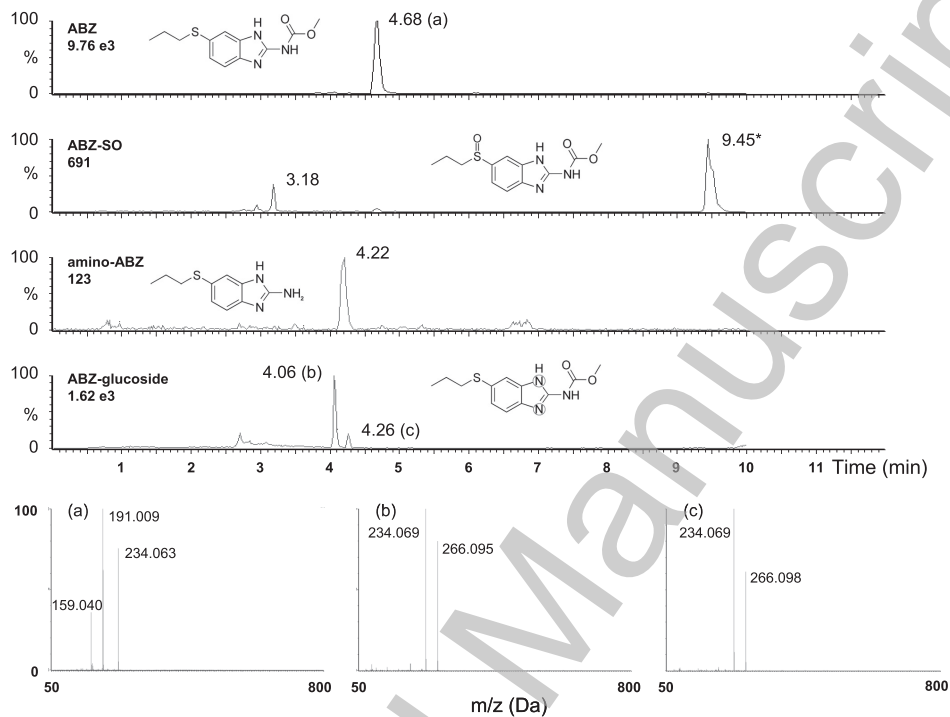
Gene ID	Gene Description	Type of reporter	GFP expression	Image
C06B3.3	<i>cyp-35C1</i>	PCR-fusion (transcriptional) AND plasmid PJM-355 (transcriptional)	intestine	
K07C6.5	<i>cyp-35A5</i>	PCR fusion (translational)	intestine (highly expressed)	
C03G6.15	<i>cyp-35A2</i>	PCR fusion (translational)	intestine (highly expressed)	
C29F7.2	Predicted small molecule kinase	PCR-fusion (transcriptional)	pharynx and posterior intestine (weak)	GFP very weak
T16G1.6	Predicted small molecule kinase	PCR-fusion (transcriptional)	anterior and posterior intestine (plus head neurones at L3)	
C04F5.7	<i>ugt-63</i>	PCR-fusion (transcriptional)	muscle (very weak expression)	GFP very weak
R03D7.6	<i>gst-5</i>	PCR fusion (translational)	intestine, pharynx and circum-pharynx neurones	
ZC443.6	<i>ugt-16</i>	PCR-fusion (transcriptional)	intestine and pharynx (highly expressed)	

Figure 5



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Figure 6

