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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

Steven T. LAING*, Al IVENS†, Roz LAING*, Sai RAVIKUMAR‡, Victoria BUTLER‡, Debra J. WOODS§ and John S. GILLEARD‡[1]

*University of Glasgow, Faculty of Veterinary Medicine, Glasgow G61 1QH, Scotland, U.K., †Fios Genomics Ltd, King's Buildings, Edinburgh EH9 3JL, Scotland, U.K., ‡University of Calgary, Faculty of Veterinary Medicine, Calgary, Alberta T2N 4N1, Canada, and §Pfizer Animal Health, Kalamazoo, MI 49001, U.S.A.

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 for 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 that the transcriptional response is dominated by genes encoding XMEs (xenobiotic-metabolizing enzymes), particularly cytochrome P450s and UGTs (UDP-glucuronosyl transferases). 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.

Key words: anthelmintic resistance, cytochrome P450, metabolism, nematode, UDP-glucuronosyl transferase.

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, metabolized 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. Previous work has shown that 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. [14] demonstrated a discreet 450 nm absorption peak in carbon-monoxide-bound, dithionate-treated *Caenorhabditis elegans* microsome preparations, supporting the presence of active cytochrome P450 protein in the nematode. However, surprisingly, nematode xenobiotic responses to and biotransformation of any of the therapeutically important anthelmintic drugs have yet to be investigated.

ABZ (albendazole) belongs to the BZ (benzimidazole) 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 BZ 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 parasite may be a mechanism of resistance [24].

Pharmacokinetic analyses have shown that ABZ, and the other BZs, are highly susceptible to biotransformation in mammals [25,26]. ABZ is almost entirely converted into the active metabolite ABZ-SO (ABZ sulfoxide) during first-pass metabolism. This reaction is thought to be primarily catalysed by flavin mono-oxygenase and the CYP3A family [27–29]. Further

Abbreviations used: ABZ, albendazole; ABZ-SO, ABZ sulfoxide; ABZ-SO_2, ABZ sulfone; BZ, benzimidazole; DAVID, Database for Annotation, Visualization and Integrated Discovery; FDR, false discovery rate; GFP, green fluorescent protein; GST, glutathione transferase; MS/MS, tandem MS; PPARα, peroxisome-proliferator-activated receptor α; QPCR, quantitative PCR; RNAi, RNA interference; UGT, UDP-glucuronosyl transferase; XME, xenobiotic-metabolizing enzyme.

[1] To whom correspondence should be addressed (email jsgillea@ucalgary.ca).

sulfoxidation to the inactive ABZ-SO_2 (ABZ sulfone) is thought to occur via the CYP1A family [27]. Like many xenobiotics, the presence of ABZ and its metabolites has been shown to induce cytochrome P450 enzymes and other XMEs (xenobiotic-metabolizing 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 ABZ because it is a therapeutically important member of the BZ 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 ABZ exposure utilizing a drug-resistant mutant to minimize responses associated with non-specific stress or with the mode of action of the drug. We have found that under these conditions, genes encoding drug-metabolizing enzymes, particularly cytochrome P450s and UGTs (UDP-glucuronosyl transferases), dominate the transcriptional response. We have also demonstrated significant metabolism of ABZ 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 ABZ 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. The present study demonstrates that the BZ drug ABZ 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

C. elegans strains

In order to minimize the transcriptomic changes caused by stress secondary to the effect of the drug, the 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 CGC (*C. elegans* Genetics Center, University of Minnesota, Minneapolis, MN, U.S.A.). 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 0.05 M K_2HPO_4, 871 ml/l 0.05 M KH_2PO_4 and 0.1 M 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-PEG [cholesterol-poly(ethylene glycol) 600 sebacate; Sigma] was used at a final concentration of 25 μg/ml. This appeared to increase the solubility of the drug compared with the use of standard cholesterol. Approx. 10000 L1 larvae were added to each of two 50 ml cultures, containing 1 ml of concentrated OP50. The worms were grown at 20 °C, with shaking at 240 rev./min for 70 h, until many young adults were present. Then, 450 μl of 20 mg/ml ABZ (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 h, 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 (quantitative PCR). These were carried out in an identical manner except for the use of a commercial preparation of ABZ (Albex 10 %, Chanelle). Investigation of gene up-regulation following exposure to a gradient of ABZ concentrations was also undertaken. Five matched cultures of *C. elegans* were prepared. Cultures were exposed to 0.3, 3, 30 and 300 μg/ml ABZ or no ABZ control for 4 h. ABZ (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 the manufacturers protocol. Briefly, pelleted worms were homogenized in 4 vol. of TRIzol® reagent, subjected to two chloroform extractions and precipitated in propan-2-ol. The RNA was then treated with RNase-free DNase I (Qiagen) in solution before purification and concentration using RNeasy columns (Qiagen). The 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. Total RNA (5 μg for each sample) was used as a 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 of TE (10 mM Tris and 1 mM EDTA) buffer and stored at -80 °C until use.

Microarray hybridization and analysis

Sample labelling 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 (September 2008). Scanned array images (CEL files) were quality-control assessed using the arrayQualityMetrics Bioconductor package (http://www.bioconductor.org) in the R environment (http://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 (http://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 an FDR (false discovery rate) cut-off of 5–10 %.

Gene ontology analysis

DAVID software (Database for Annotation, Visualization 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 an FDR of less than 10 %. Prevalence of annotation terms within the list of differentially expressed genes were compared with the prevalence in the whole *C. elegans* genome. Fold-enrichment was calculated and a modified Fisher's exact test (EASE score) was used to assign significance. Gene functional classification clustering was carried out using medium stringency.

Real-time QPCR

Relative quantification 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 normalizing gene. This constitutively expressed gene showed no significant changes on microarray analysis and has been extensively used as a normalizing gene in differential expression studies in *C. elegans* [43]. 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 S1 at http://www.BiochemJ.org/bj/432/bj4320505add.htm. The final concentration of primers was between 300 and 400 nM in a total reaction volume of 25 μl.

Expression pattern analysis

GFP (green fluorescent protein) 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 [44]. The putative promoter region, 3 kb upstream from the ATG start site of the gene of interest, was fused to the *gfp* gene, including synthetic introns, and the *unc-54* 3′ UTR (untranslated region) 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 (a gift from Dr Jim McGhee, University of Calgary, Faculty of Medicine, Alberta, Canada). 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 Improvision Openlab software (http://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 and 240 rev./min. One of the paired cultures was killed by heating to 50 °C for 30 min in a waterbath. ABZ was added to both the experimental and heat-killed culture to a final concentration of 15 μg/ml. Following 7 h of culture with ABZ the worms were harvested by centrifugation at 1000 ***g*** at 4 °C in M9 buffer and washed three times in M9 buffer. Packed worms were homogenized first on ice using 3×30 s cycles at 1 min intervals with an Ultra Turrax T8 homogenizer (IKA-Werke) at maximum speed followed by 3×30 s cycles with 1 min intervals of sonication (maximum amplitude, 16 microns) with a Soniprep 150 (Sanyo). ABZ and metabolites were extracted in 10 vol. of methanol/Tris (pH 9; ratio 9:1). The samples were centrifuged at 2500 ***g*** at 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 μl of 50:50 acetonitrile/deionized MQ (milli-Q) water supplemented with 0.1 % formic acid and centrifuged at 2500 ***g*** to remove solids. Bacterial controls were carried out in a similar manner using cultures containing no nematodes and 3 ml of concentrated OP50 suspension per 250 ml culture.

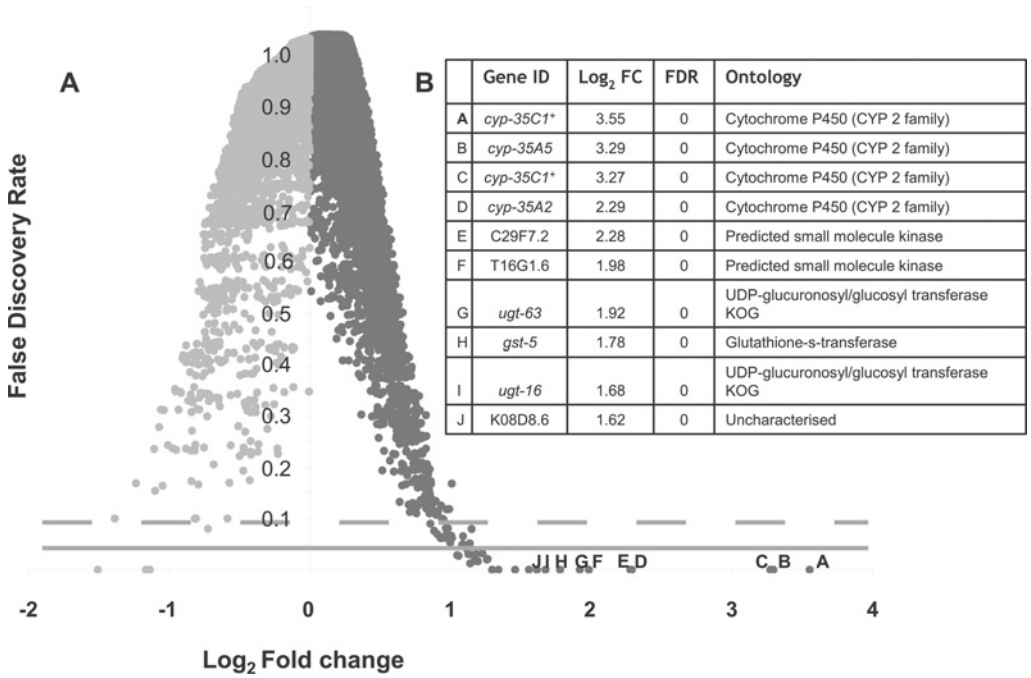
Resuspended residue (5 μl) was injected on to a Waters HSS 1.8 μm C_{18} column (100 mm×1 mm), using a Waters Acquity Ultra Performance LC system. In the mobile phase, a gradient of 95:5 % water/acetonitrile+0.1 % formic acid to 100 % acetonitrile+0.1 % formic acid over 12 min, was applied to the column at 200 μl/min. MS/MS (tandem MS) analysis was carried out using a Micromass MS Technologies Q-Tof Premier™. A reference spray using leucine enkephalin was used to provide accurate mass data. A standard solution of ABZ was analysed alongside experimental samples and mass readings were correct to within ±16 p.p.m. (0.004 Da). Analysis was carried out by Q1 and MS/MS methods using Mass Lynx 4.1 software (Waters).

RESULTS

Characterization of the transcriptional response to ABZ using whole-genome microarrays

Three biological replicates of *C. elegans* exposed to 300 μg/ml ABZ for 4 h were prepared along with paired negative controls. Total RNA was extracted and hybridized to Affymetrix *C. elegans* arrays. The majority of genes showed 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 an FDR of 5 %. Further analysis focused 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 S2 at http://www.BiochemJ.org/bj/432/bj4320505add.htm 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 software. This data set contained 51 probesets, which represented 42 genes in the *C. elegans* genome. There was enrichment of several gene ontology terms associated with transferase and mono-oxygenase enzymes (Figure 2). These classes of enzyme are common within xenobiotic metabolism pathways. In total 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 (Kyoto Encyclopedia of Genes and Genomes) pathway terms were assessed in addition to gene ontology terms. Using this method only six genes were not annotated, all of which were uncharacterized 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 UGT 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



	Gene ID	Log_2 FC	FDR	Ontology
A	*cyp-35C1*+	3.55	0	Cytochrome P450 (CYP 2 family)
B	*cyp-35A5*	3.29	0	Cytochrome P450 (CYP 2 family)
C	*cyp-35C1*+	3.27	0	Cytochrome P450 (CYP 2 family)
D	*cyp-35A2*	2.29	0	Cytochrome P450 (CYP 2 family)
E	C29F7.2	2.28	0	Predicted small molecule kinase
F	T16G1.6	1.98	0	Predicted small molecule kinase
G	*ugt-63*	1.92	0	UDP-glucuronosyl/glucosyl transferase KOG
H	*gst-5*	1.78	0	Glutathione-s-transferase
I	*ugt-16*	1.68	0	UDP-glucuronosyl/glucosyl transferase KOG
J	K08D8.6	1.62	0	Uncharacterised

Figure 1 Volcano plot representing all the probesets on the *C. elegans* Genechip

(**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 an FDR $<5\%$. Those below the broken line showed significant changes in expression with an FDR $<10\%$ (**B**) The top ten up-regulated genes following exposure to ABZ. Note the large number of genes belonging to potential XME families. *cyp-35C1*+ is represented by two probes on the Genechip and appears twice in the top ten.

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 XMEs, including an alcohol dehydrogenase and a GST (glutathione transferase) (Supplementary Table S2). Other up-regulated genes include predicted small molecule kinases and *jnk-1* (c-Jun N-terminal kinase 1), another kinase likely to be 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 (S.T. Laing, A. Ivens and J.S. Gilleard, unpublished work). 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 coincidently up-regulated with *cyp-35A5, gst-5* and *vem-1* in response to infection with the toxin-secreting bacterium *Pseudomonas aeruginosa* (Supplementary Table S2) [48].

The ten probesets showing the greatest up-regulation represent a putative xenobiotic metabolism pathway

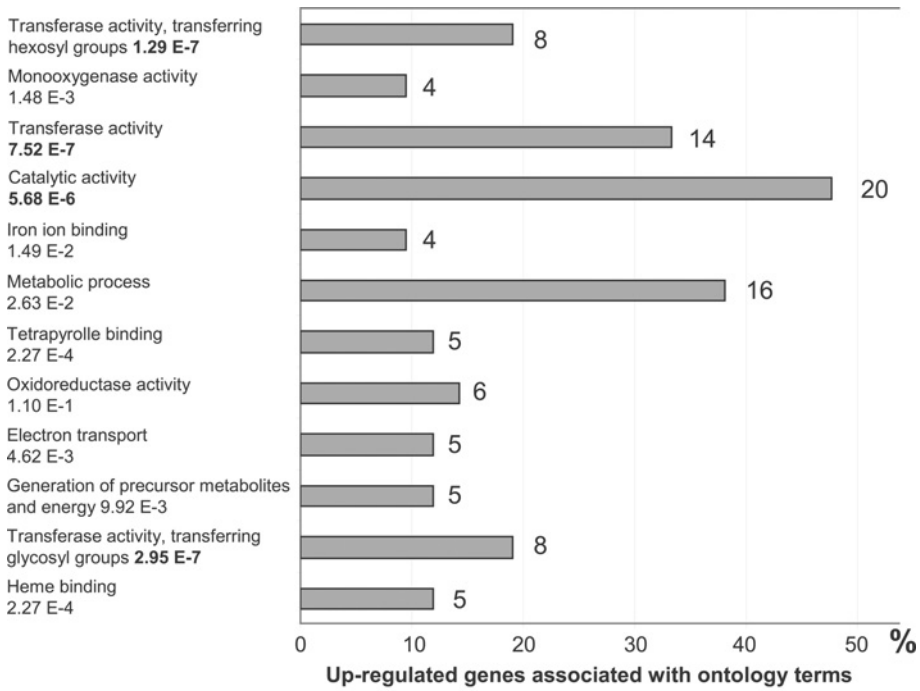
The top ten gene list, based on log_2 fold change, includes three *cyps*, two *ugts* and a *gst* (Figure 1B). Of the three probesets not representing genes encoding xenobiotic-metabolizing proteins, only one is completely uncharacterized. The other two both represent predicted small molecule kinases, which could potentially be involved in the signalling cascade in response to ABZ. Up-regulation of all of these genes in response to ABZ exposure was confirmed using real-time QPCR (Figure 3A). The fold-change of specific genes was higher using real-time 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 real-time QPCR analysis, in addition to the normalizing 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 real-time QPCR technique. Similarly *cyp-37B1*, which we have found to be up-regulated in response to ivermectin exposure (S.T. Laing, A. Ivens and J.S. Gilleard, unpublished work), was not significantly up-regulated following ABZ exposure using microarray analysis or real-time QPCR.

cyp-35C1, cyp-35A5, cyp-35A2 and ugt-16 respond to ABZ in concentration dependent manner

Real-time 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 h (Figure 3B). While *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 h 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



Functional classification group 1

ID	Description
ugt-1	UDP-glucuronosyl/ glucosyl transferase KOG
Y43D4A.2	UDP-glucuronosyl/ glucosyl transferase protein domain
ugt-5	UDP-glucuronosyl/ glucosyl transferase KOG
ugt-16	UDP-glucuronosyl/ glucosyl transferase KOG
ugt-25	UDP-glucuronosyl/ glucosyl transferase KOG
ugt-41	UDP-glucuronosyl/ glucosyl transferase KOG
ugt-63	UDP-glucuronosyl/ glucosyl transferase KOG
ugt-22	UDP-glucuronosyl/ glucosyl transferase KOG
ugt-1	UDP-glucuronosyl/ glucosyl transferase KOG

Functional classification group 2

ID	Description
cyp-35A2	cytochrome P450
cyp-35C1	cytochrome P450
cyp-35A5	cytochrome P450
cyp-29A2	cytochrome P450
vem-1	Putative steroid membrane receptor KOG

Figure 2 Ontology terms associated with genes up-regulated in response to 4 h exposure of strain CB3474 to 300 μg/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 (a total of 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 UGTs and a cluster of P450-encoding genes.

proposed as the main site of detoxification in *C. elegans* [51]. *gfp* expression under the control of the *ugt-63* promoter was localized to the hypodermis.

Demonstration of ABZ metabolism in *C. elegans* and the production of novel glucoside metabolites

Organically extracted homogenates of *C. elegans* following exposure to ABZ for 7 h were initially analysed using a total ion scan with accurate mass analysis. As well as an intense peak for ABZ at approx. 4.68 min, there were also significant peaks for ABZ-SO (mass 282.091 Da, elution time 3.18 min), 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 $COOCH_3$) 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 ABZ were unique to the experimental live worm samples and their identities were confirmed using MS/MS fragment analysis (Figures 5 and 6). Fragmentation of ABZ typically reveals three major fragment ions: 234.06 Da, 191.01 Da and 159.04 Da. Fragmentation of the proposed glucose conjugates of ABZ identified only two major fragments. One of

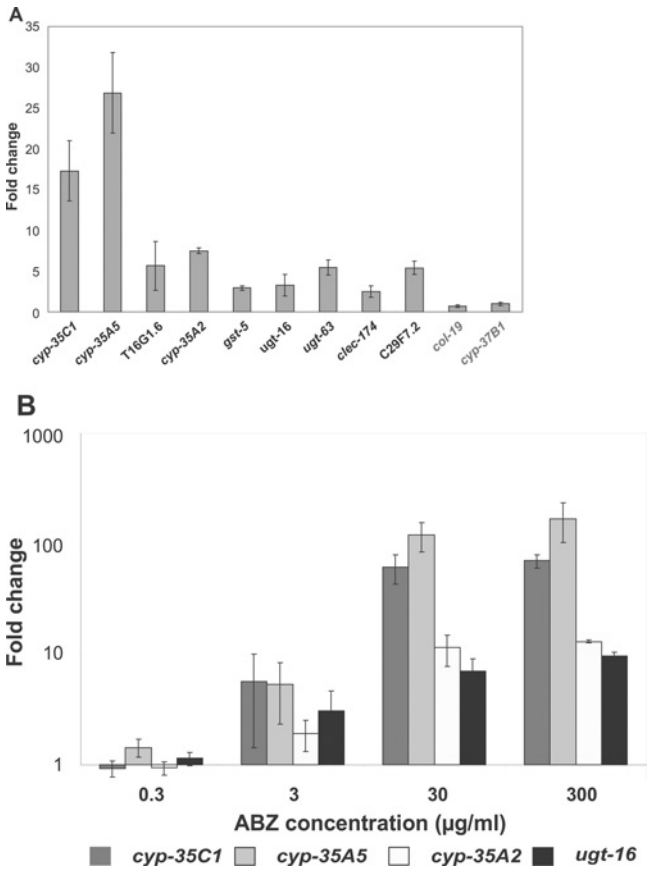


Figure 3 Real-time QPCR confirmation of the ABZ-responsive genes identified in the microarray

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

mass 266.09 Da is proposed to be ABZ itself and the other of mass 234.06 Da is likely to be the ABZ acylium, caused by protonation of the carbamate, which is also present in the ABZ fragment ion spectrum. These findings confirm the identity of the peaks of mass 428.149 Da as metabolites of ABZ and the mass change is consistent with them being glucose conjugates. The fragment ion spectra for each of the glucoside metabolites are identical. Therefore while the molecular position at which the glucose has been conjugated is not certain, it is likely that the conjugation sites are the two BZ nitrogens, which are the same number of bonds from the sulfur and the carbamate ester and share the double bond due to resonance. Interestingly, there were no peaks consistent with ABZ-SO_2 (expected mass 298.09 Da) or glucuronidation of ABZ (expected mass 442.09 Da).

The peak intensity of the ABZ-glucoside metabolites were between 0.7 % and 16 % of that of ABZ in the analysed incubations. The group of cultures that were pre-exposed to the PPARα (peroxisome-proliferator-activated receptor α) agonist fenofibrate, a potent inducer of cytochrome P450s, UGTs and several other XMEs, produced a more intense ABZ-glucoside peak (Figure 6). Although accurate quantification of the metabolites was not possible since an ABZ-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 BZ-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 300 μg/ml ABZ for 4 h [52–57]. This, together with the fact that such a small number of genes had significant changes in expression level, suggests that the up-regulated genes we have identified represent a specific xenobiotic response to ABZ exposure. Within this subset of genes, only the cytochrome P450 and UGT families were significantly enriched. In addition, concentration-gradient experiments using real-time QPCR 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 ABZ-SO 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 ABZ 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 (S.T. Laing, A. Ivens and J.S. Gilleard, unpublished work). The CYP35 family in *C. elegans* is most closely related to the CYP2 family of humans and other mammals. CYP35C1 has closest homology with *Homo sapiens* CYP2B6 (BLASTp E-value: 1.1 e-56, 93.9 % length). Both CYP35A5 and CYP35A2 bear closest homology with *H. sapiens* CYP2C8 (BLASTp E-value: 4.2 e-53, 93.9 % length and 4.6 e-57, 97 % length respectively). Whereas these do not appear to be the major CYP isoforms involved in ABZ 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 ten up-regulated genes following ABZ exposure, the predicted polypeptide encoded by *ugt-63* has closest homology with mammalian UGT1A1 (BLASTp E-value: 1.7 e-27, 95.7 % length) and that of *ugt-16* has closest homology with 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 up 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]. Although 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.

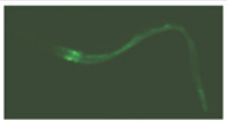
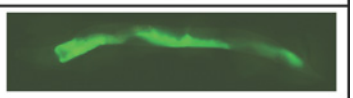
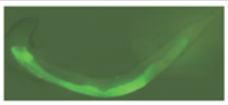
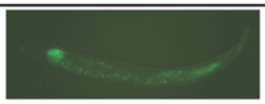
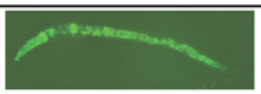
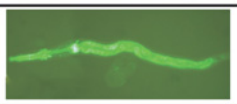
Gene ID	Gene Description	Type of reporter	GFP expression	Image
C06B3.3	*cyp-35C1*	PCR-fusion (transcriptional) AND plasmid PJM-355 (transcriptional)	intestine	
K07C6.5	*cyp-35A5*	PCR fusion (translational)	intestine (highly expressed)	
C03G6.15	*cyp-35A2*	PCR fusion (translational)	intestine (highly 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	*ugt-63*	PCR-fusion (transcriptional)	muscle (very weak expression)	GFP very weak
R03D7.6	*gst-5*	PCR fusion (translational)	intestine, pharynx and circum-pharynx neurones	
ZC443.6	*ugt-16*	PCR-fusion (transcriptional)	intestine and pharynx (highly expressed)	

Figure 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 shown.

Following incubation with *C. elegans* cultures, ABZ was shown to be metabolized to produce ABZ-SO and two ABZ-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 sulfoxidation. This metabolite, which is the major ABZ 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 ABZ metabolism [25,26,32,66]. Hence the metabolism of ABZ by nematodes differs significantly from that in mammals and involves the production of what could be nematode-specific metabolites. A recent study reported that ABZ-SO and two ABZ-glucoside conjugates, similar to those we identified in the present study 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 that the biotransformation pathways may be conserved in the strongylid nematodes, which includes many important human and animal parasites. This is an exciting result as it suggests that the dissection of the ABZ metabolism pathway in *C. elegans* will be of relevance to strongylid 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 mono-oxygenase 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 ABZ biotransformation reactions in *C. elegans*. This is supported by the experiment in which prior exposure of *C. elegans* to the PPARα 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 ABZ exposure are strong candidates for the key catalytic enzymes involved in ABZ-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

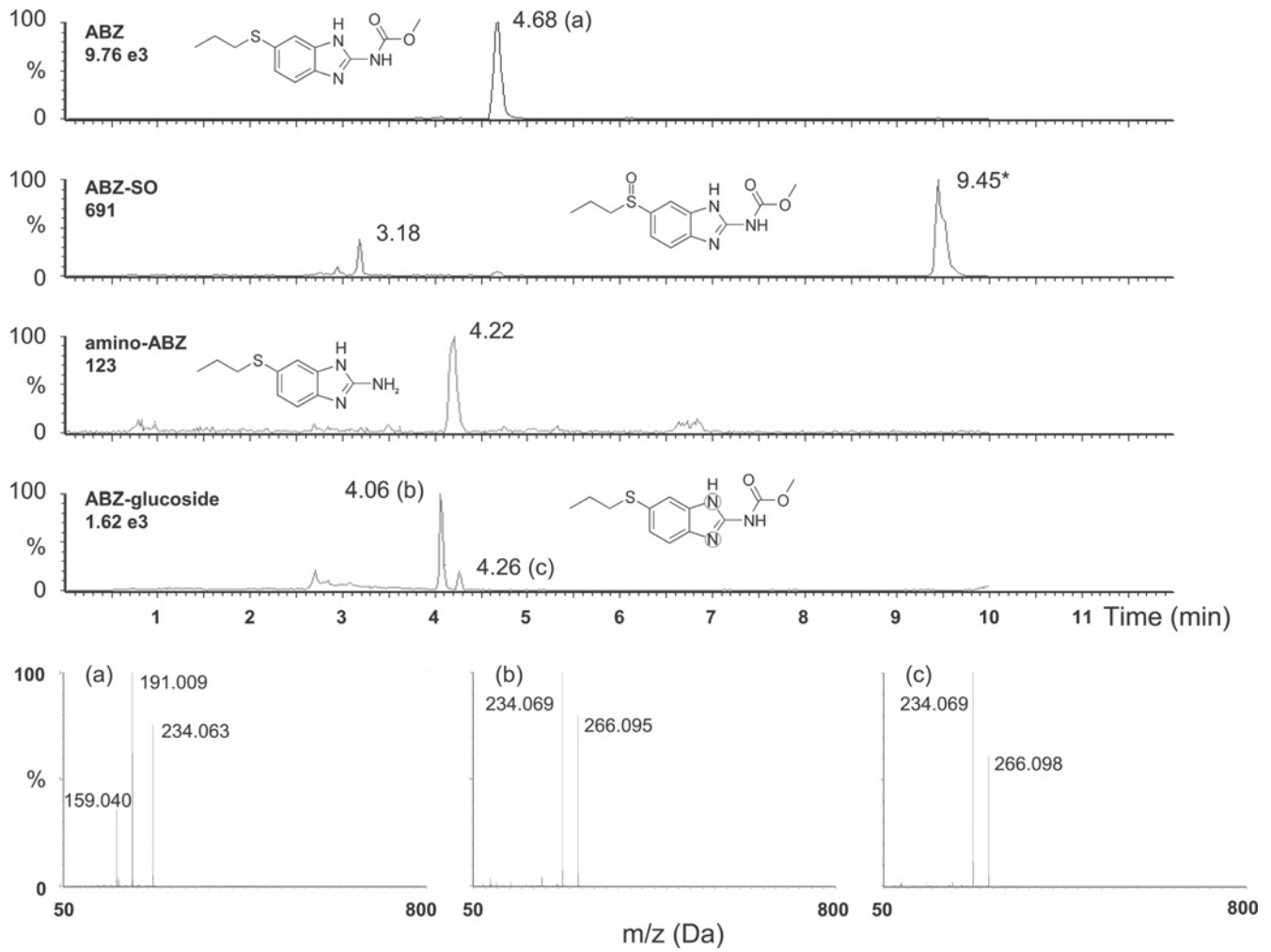


Figure 5 Chromatograms of ABZ and metabolites from *ex vivo C. elegans* incubation

Intensity (counts per s) of the peaks of interest is shown in the top left-hand corner of each chromatogram and the proposed structures are overlayed. The scanned masses were: ABZ 266.093 Da, ABZ-SO 282.091 Da, amino-ABZ 208.092 Da and ABZ-glucoside 428.149 Da. The *ex vivo* incubations showed intense peaks for ABZ-SO, amino-ABZ and ABZ-glucoside, but the 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 ABZ. The exact structures of these metabolites remain unknown, but the nitrogen atoms that are the likely to be sites of glucose conjugation are highlighted. The * indicates a peak at approx. 9.45 min with the same mass as ABZ-SO (282.088 Da) present in *ex vivo* incubations. MS/MS studies confirmed this was not a metabolite of ABZ.

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 ABZ may provide some clues as to the mechanism of induction. Many of the genes up-regulated in response to ABZ, including *cyp* and *ugt* genes have been proposed as regulatory targets of MDT-15 (Supplementary Table S3 at http://www.BiochemJ.org/bj/432/bj4320505add.htm). MDT-15 is a mediator subunit, an evolutionarily 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. [72] investigated the targets of the product of *mdt-15* by using whole-genome microarrays to compare the transcriptomes of *mdt-15(RNAi)* (where RNAi is RNA interference) worms to a control population. Of the 42 genes up-regulated in response to ABZ 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 S3). Conspicuously, eight of the top ten 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, the present 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 BZ drug ABZ, and does so in a completely novel manner compared with any mammal thus far investigated. In addition, the present study provides a platform upon which to investigate the xenobiotic response to other anthelmintics, to functionally investigate the role of parasitic nematode XMEs in anthelmintic metabolism and to further investigate the role of nuclear hormone receptors in xenobiotic responses in nematodes.

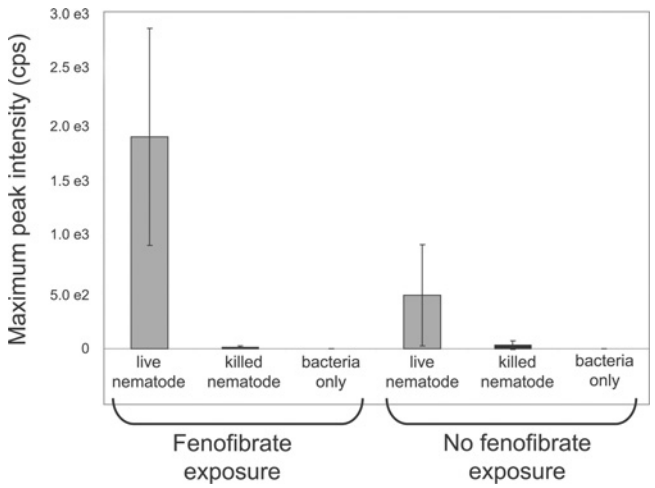


Figure 6 Relative intensity of ABZ-glucoside metabolite (elution time 4.06 min) from cultures with and without pre-exposure to fenofibrate (±S.E.M.)

ABZ-glucoside production is greater following pre-exposure of worm cultures to 20 μg/ml fenofibrate. Note the lack of an ABZ-glucoside peak in the bacterial control group. The histogram represents the results of three biological replicates for each condition.

AUTHOR CONTRIBUTION

Steven Laing undertook the drug exposure experiments, prepared RNA and undertook the ontological analysis of the microarray data. He also undertook the real-time QPCR and MS experiments and created several *C. elegans* transgenic lines. Al Ivens carried out the microarray hybridization, normalization of the data and preliminary analysis. Roz Laing assisted with microarray ontological analysis. Sai Ravikumar and Victoria Butler constructed several of the *C. elegans* transgenic lines. Debra Woods supervised the MS experimentation. John Gilleard conceived, planned and supervised the experiments, assisted with the interpretation of data and managed the project. The manuscript was written by Steven Laing and John Gilleard.

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online data

SUPPLEMENTARY ONLINE DATA

Characterization of the xenobiotic response of *Caenorhabditis elegans* to the anthelmintic drug albendazole and the identification of novel drug glucoside metabolites

Steven T. LAING*, Al IVENS†, Roz LAING*, Sai RAVIKUMAR‡, Victoria BUTLER‡, Debra J. WOODS§ and John S. GILLEARD‡[1]

*University of Glasgow, Faculty of Veterinary Medicine, Glasgow G61 1QH, Scotland, U.K., †Fios Genomics Ltd, King's Buildings, Edinburgh EH9 3JL, Scotland, U.K., ‡University of Calgary, Faculty of Veterinary Medicine, Calgary, Alberta T2N 4N1, Canada, and §Pfizer Animal Health, Kalamazoo, MI 49001, U.S.A.

Table S1 Primer sequences used in the present study

Gene	Forward primer sequence	Reverse primer sequence
ama-1	5′-AAGCGGCTCACAATGATCTACGA-3′	5′-ACACGGCGGTATGATGGTTGA-3′
clec-174	5′-CGTTTGCCCAGTCGGTAATGA-3′	5′-ACCGGACGATAATGGCAAGAAT-3′
col-19	5′-GTTCCAGGATGGTATGGTTGAATTAGAGCT-3′	5′-GGTCCGCAGTTACATTGCTCGAATCC-3′
cyp-35A2	5′-ATGACTGCACCCGTTTGGTTT-3′	5′-ACGCGTCAGTGTAATCTTGCA-3′
cyp-35A5	5′-AAAAGGTTATCCCATTCGGAGTT-3′	5′-AACGCTCTCTTTGCAATACTGTA-3′
cyp-35C1	5′-GAGATTTGATGGAGGAGAAGATT-3′	5′-CATCAAATCGAAATCCTAAGAGCA-3′
cyp-37B1	5_-AAGAACGGTGGAGCAGGATGT-3_	5′-TTCGGGGTCCAGCACTAATG-3′
C29F7.2	5′-CGGAGTTAGGGTACATGTCAA-3′	5′-CAACATTAGCAGAGTGGTCAGTT-3′
gst-5	5′-CCGGACAACAATACGAGGAT-3′	5′-GCGGTTTTTCCGTTGAGCTT-3′
T16G1.6	5′-GGGAATGGAATATGTCGATGAT-3′	5′-CTTTTAGACCATCGTCGTTGAA-3′
ugt-16	5′-TGCATCAATGCCGGAAACTACTT-3′	5′-TTCCAAGCCCTCCGTGAGTT-3′
ugt-63	5′-CGCCAGGACATTGATTTTGGAA-3′	5′-ACGGTGCTTCAGGATGTTGTT-3′

[1] To whom correspondence should be addressed (email jsgillea@ucalgary.ca).

Table S2 Significantly up-regulated and down-regulated genes (FDR < 10%) following exposure of *C. elegans* to ABZ

Items in bold represent genes that were similarly regulated in response to infection with *Pseudomonas aeruginosa* in the study by Shapira et al. [1] . *P. aeruginosa* secretes a potent toxin to which an XME response would be expected. *Ten of the genes contain a CUB-like domain, which is of unknown function. Please note that six probesets considered to have significant up-regulation are not included as these represent oligonucleotides that have not been mapped to a specific gene due to gene models changing since the inception of the microarray.

Affymetrix ID	Gene ID	Log_2 FC	FDR
Up-regulated genes			
181334_s_at	C27H5.4	1.01	0.067
177487_at	**C29F3.7***	1.12	0.043
178316_at	C29F7.2	2.28	0.000
182733_at	C31A11.5	0.97	0.081
183052_at	**C32H11.1***	1.26	0.020
180909_at	**C32H11.4***	1.14	0.013
183115_at	**C34H4.2**	0.88	0.076
172184_x_at	*clec-174*	1.46	0.000
189350_at	*cyp-29A2*	1.13	0.031
189512_at	*cyp-35A2*	2.29	0.000
189394_at	***cyp-35A5***	3.29	0.000
189282_at	*cyp-35C1*	3.55	0.000
189283_s_at	*cyp-35C1*	3.27	0.000
188882_at	*dhs-23*	1.18	0.030
184863_s_at	***dod-24****	0.91	0.070
178843_at	**F08G5.6***	1.14	0.052
189525_s_at	F13H6.3	1.46	0.000
179511_at	**F20G2.5***	1.09	0.050
179695_at	**F35E12.5***	1.26	0.021
179914_at	**F35E12.8***	0.92	0.064
180315_at	**F44G3.10**	0.86	0.090
180925_at	**F49F1.1**	1.12	0.041
181132_s_at	F49F1.7	1.01	0.062
181635_at	F55G11.2*	0.97	0.064
172594_x_at	*fbxa-60*	0.97	0.068
191071_at	*gst-21*	1.18	0.018
192820_at	*gst-5*	1.78	0.000
175640_at	*jnk-1*	1.05	0.029
177701_s_at	**K08D8.6***	1.62	0.000
177700_at	**K08D8.6***	1.34	0.000
178425_at	**K08D8.6***	1.16	0.029
189646_at	K09C4.5	0.93	0.062
172744_at	*mtl-1*	1.00	0.065
188930_at	T10B5.8	1.56	0.000
178563_at	T16G1.6	1.98	0.000
178297_at	**T24B8.5**	0.95	0.098
190879_at	*ugt-1*	1.23	0.032
191418_at	*ugt-16*	1.68	0.000
193604_at	*ugt-22*	1.30	0.000
184602_at	*ugt-25*	1.15	0.080
190849_at	*ugt-41*	1.09	0.066
191066_s_at	*ugt-5*	0.96	0.063
190744_at	*ugt-63*	1.92	0.000
188031_s_at	***vem-1***	1.19	0.017
183703_s_at	Y43D4A.2	1.08	0.048
Down-regulated genes			
183330_s_at	C09B8.4	− 1.51	0.000
190958_s_at	F44E5.4	− 1.16	0.000
171941_s_at	F44E5.5	− 1.14	0.000
182428_s_at	*lys-3*	− 0.72	0.080
188822_at	***acdh-1***	− 1.39	0.100
191623_at	*ugt-53*	− 0.82	0.100
190646_s_at	**F18E3.7**	− 0.81	0.100
183676_s_at	*numr-1*	− 0.58	0.100

Table S3 Log_2FC of genes with significantly changed expression level following ABZ exposure, that were also represented in the *mdt15(RNAi)* experiment of Taubert et al. [2], are compared between each of the experiments

Genes that are likely to be regulatory targets of MDT-15, will have a negative fold-change in the Log FC *mdt-15(RNAi)* column.

Gene ID	Log FC *mdt-15(RNAi)*	Log FC ABZ exposure
C27H5.4	− 1.576	1.006
C29F3.7	− 1.289	1.122
C29F7.2	− 2.347	2.277
C31A11.5	− 0.206	0.967
C32H11.4	1.134	1.14
C34H4.2	0.478	0.885
clec-174	0.037	1.459
cyp-29A2	− 2.693	1.127
cyp35A2	− 1.8	2.288
cyp-35A5	− 1.549	3.294
cyp-35C1	− 2.862	3.552
dhs-23	1.613	1.183
dod-24	1.87	0.906
F08G5.6	− 2.323	1.137
F13H6.3	− 0.447	1.459
F20G2.5	0.034	1.087
F35E12.5	− 1.434	1.264
F35E12.8	0.277	0.924
F44G3.10	− 0.155	0.856
F49F1.1	− 0.007	1.117
F49F1.7	− 0.547	1.01
F55G11.2	0.687	0.965
fbxa-60	0.716	0.968
gst-21	− 1.04	1.176
gst-5	− 0.86	1.778
K08D8.6	− 0.375	1.615
K09C4.5	− 1.288	0.931
K11G9.6	0.645	0.998
T10B5.8	− 1.436	1.557
T16G1.6	− 3.698	1.983
T24B8.5	− 3.5	0.95
ugt-1	− 2.396	1.227
ugt-22	− 1.147	1.295
ugt-25	− 1.782	1.151
ugt-41	− 0.953	1.089
ugt-63	− 3.149	1.918
vem-1	− 0.182	1.195
Y43D4A.2	− 0.269	1.082
numr-1	1.136	− 0.584
lys-3	0.377	− 0.724
ugt-53	− 4.885	− 0.817

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