Similarities and differences in the biotransformation and transcriptomic responses of *Caenorhabditis elegans* and *Haemonchus contortus* to five different benzimidazole drugs

S.J. Stasiuk\(^a\), G. MacNevin\(^b\), M.L. Workentine\(^c\), D. Gray\(^b\), E. Redman\(^b\), D. Bartley\(^c\), A. Morrison\(^c\), N. Sharma\(^a\), D. Colwell\(^b\), D.K. Ro\(^a\), J.S. Gilleard\(^a\*,\)

\(^a\) Faculty of Veterinary Medicine, University of Calgary, Calgary, Alberta, T2N 4N1, Canada
\(^b\) Agriculture and Agri-Food Canada, Lethbridge Research Station, 5403 1st Ave South, Lethbridge, Alberta, Canada, T1J 4B1
\(^c\) Moredun Research Institute, Pentlands Science Park, Edinburgh, EH26 0PZ, UK

**A R T I C L E  I N F O**

Keywords:
*Caenorhabditis elegans*
*Haemonchus contortus*
Benzimidazoles
Xenobiotic metabolism
Transcriptomic
LC/MS/MS

**A B S T R A C T**

We have undertaken a detailed analysis of the biotransformation of five of the most therapeutically important benzimidazole anthelmintics - albendazole (ABZ), mebendazole (MBZ), thiabendazole (TBZ), oxendazole (OxBZ) and fenbendazole (FBZ) - in *Caenorhabditis elegans* and the ruminant parasite *Haemonchus contortus*. Drug metabolites were detected by LC-MS/MS analysis in supernatants of *C. elegans* cultures with a hexose conjugate, most likely glucose, dominating for all five drugs. This work adds to a growing body of evidence that glucose conjugation is a major pathway of xenobiotic metabolism in nematodes and may be a target for enhancement of anthelmintic potency. Consistent with this, we found that biotransformation of albendazole by *C. elegans* reduced drug potency. Glucose metabolite production by *C. elegans* was reduced in the presence of the pharmacological inhibitor chrysin suggesting that UDP-glucuronosyl/glucosyl transferase (UGT) enzymes may catalyze benzimidazole glucosidation. Similar glucoside metabolites were detected following ex vivo culture of adult *Haemonchus contortus*. As a step towards identifying nematode enzymes potentially responsible for benzimidazole biotransformation, we characterised the transcriptomic response to each of the benzimidazole drugs using the *C. elegans* resistant strain CB3474 ben-1(e1880)III. In the case of albendazole, mebendazole, thiabendazole, and oxendazole the shared transcriptomic response was dominated by the up-regulation of classical xenobiotic response genes including a shared group of UGT enzymes (ugt-14/25/33/34/37/41/8/9). In the case of fenbendazole, a much greater number of genes were up-regulated, as well as developmental and brood size effects suggesting the presence of secondary drug targets in addition to BEN-1. The transcriptional xenobiotic response of a multiply resistant *H. contortus* strain UGA/2004 was essentially undetectable in the adult stage but present in the L3 infective stage, albeit more muted than *C. elegans*. This suggests that xenobiotic responses may be less efficient in stages of parasitic nematodes that reside in the host compared with the free-living stages.

1. Introduction

Metabolism of drugs can have a major effect on drug potency (Daborn et al., 2002; Li et al., 2007; Amenya et al., 2008), for example, in humans, the cytochrome P450s and UDP-glucuronosyltransferases together are responsible for clearing greater than 90% of all commercial drugs; CYP3A4, with its large active site, is responsible for metabolizing 30% of all commercial drugs tested (Rowland et al., 2013; Zanger and Schwab, 2013; Mazerska et al., 2016). Understanding anthelmintic metabolism and the enzyme pathways involved in gastrointestinal parasites should illuminate our understanding of factors that affect anthelmintic potency and may lead to the development of synergistic compounds that increase the potency of anthelmintics currently in use. Use of detoxifying enzyme inhibitors in gastrointestinal parasitic nematodes has been shown to increase anthelmintic drug potency, suggesting that xenobiotic metabolism occurs in these parasites. In the cattle parasites *Cooperia oncophora* and *Ostertagia ostertagi*, the use of the cytochrome P450 inhibitor, piperonyl butoxide made the larvae of both strains more sensitive to anthelmintics suggesting that cytochrome P450s play a role in metabolizing thiabendazole and macrocyclic lactones such as Ivermectin (AlGusbi et al., 2014). The P-glycoprotein inhibitor, verapamil, increased the susceptibility of benzimidazole

https://doi.org/10.1016/j.ijpddr.2019.09.001
Received 18 May 2019; Received in revised form 5 September 2019; Accepted 8 September 2019
Available online 09 September 2019
2211-3207/ © 2019 Published by Elsevier Ltd on behalf of Australian Society for Parasitology. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/BY-NC-ND/4.0/).
resistant strains to thiabendazole, and increased the susceptibility of *O. ostertagi* strains to ivermectin (AIGusbi et al., 2014). In the sheep parasite, *H. contortus*, the use of UGT inhibitor, 5-nitrocoumaril was able to make naftalophos resistant worms more sensitive in a Larval Developmental Assay (LDA), and also increased the sensitivity of wild type worms to this anthelmintic (Kotze et al., 2014). Hence, there is an increasing amount of data to suggest that xenobiotic responses play an important role in modulating drug sensitivity and resistance in parasitic nematodes.

The benzimidazoles (BZ) are a family of broad-spectrum anthelmintics used in livestock, and are one of the few drugs available for the control of helmint parasites in humans (Albonico et al., 2003; Kaplan, 2004; Kaplan et al., 2004; Wolstenholme et al., 2004; von Samson-Himmelstjerna and Blackhall, 2005; Gilleard, 2006; Osei-Atweneboana et al., 2007). Although mutations in the β-tubulin drug target are clearly important (Kwa et al., 1995; Grant and Mascord, 1996; Silvestre and Humbert, 2000; Winterrowd et al., 2003; Alvarez-Sanchez et al., 2005; de Lourdes Mottier and Prichard, 2008; Hodgkinson et al., 2008), there is some evidence that other mechanisms of resistance may occur. For example, natural field populations of *C. elegans* exist in which there are several major QTL underlying benzimidazole resistance in addition to the ben-1 locus (Zamanian et al., 2017, 2018). Further, different field isolates of *H. contortus*, which have the same frequency of the isotype-1 β-tubulin resistance mutations have very different levels of drug resistance as measured by egg hatch assay L50s (von Samson-Himmelstjerna et al., 2009). Also, benzimidazole resistant *H. contortus* strains have been reported to produce increased amounts of glucosidated metabolite products (Cvilink et al., 2008a, 2008b; Vokral et al., 2012, 2013).

Benzimidazoles are good substrates for biotransformation in a large number of organisms from mammals to nematodes (Velik et al., 2005; Laing et al., 2010; Aksit et al., 2015). There is also some evidence that xenobiotic biotransformation plays a role in sensitivity and resistance of parasitic helminths to the benzimidazole drugs. In an *in vitro* assay that examined oxidation of albendazole, the resistant strains of *F. hepatica* have been shown to produce more of the oxidized product than sensitive strains (Solana et al., 2001). The liver fluke *D. dendriticum* was found to biotransform albendazole to albendazole sulphoxide, and mebendazole and flubendazole were either reduced, or reduced and methylated (Cvilink et al., 2009a, 2009b). *F. hepatica* metabolizes tri-clabendazole (TCBZ) into sulfoxide (TCBZ-SO) and sulphone (TCBZ-SO2), these metabolized products were found at higher concentrations in resistance strains (Solana et al., 2001; Mottier et al., 2004; Robinson et al., 2004). The cestode, *Moniezia expansa*, biotransforms flubendazole and mebendazole by reduction and albendazole was oxidized to albendazole sulfoxide and sulphone (Prchal et al., 2015). The parasitic nematodes, *Ascaris suum* and *Haemonchus contortus*, have also been shown to biotransform several benzimidazoles. In an *in vitro* assay, *A. suum* produced a reduced metabolite (Solana et al., 2001). *H. contortus* has been shown to produce a variety of benzimidazole metabolites that are either reduced, hydrolyzed and/or glucosidated (Cvilink et al., 2008a, 2008b; Vokral et al., 2012, 2013; Stuchlikova et al., 2018) with resistant strains producing more of these metabolized product than the sensitive strains (Vokral et al., 2012, 2013; Stuchlikova et al., 2018).

Investigation of the molecular processes involved in metabolism requires a model more tractable than parasitic nematodes, which are notoriously difficult to perform functional analysis on (Burglin et al., 1998; Geary and Thompson, 2001; Gilleard, 2004; Gilleard et al., 2005). This raises the question – is the free living nematode model, *Caenorhabditis elegans*, a good model for the study of anthelmintic metabolism of parasitic species of nematode (Gilleard, 2004; Sommer and Streit, 2011; Burns et al., 2015; Ward, 2015)? We previously found that *C. elegans* exposed to the benzimidazole, albendazole, resulted in up-regulation of several Phase I and Phase II metabolizing genes, and that albendazole is metabolized to an albendazole-hexose derivative (Laing et al., 2010). It has also been reported that there was a glucose metabolite produced and up-regulation of UDP-glucosyltransferase enzyme activity in resistant strains of *H. contortus* (Cvilink et al., 2008b; Vokral et al., 2013).

In this paper, we undertake a detailed comparison of the bio-transformation and xenobiotic responses to five of the most important benzimidazoles drugs used therapeutically in humans and animals; albendazole (ABZ), mebendazole (MBZ), thiabendazole (TBZ), oxendazole (OxBZ) and fenbendazole (FBZ) (Supplemental Figure 1). We show that they are metabolized to similar products in both *C. elegans* and *H. contortus* by glucosidation, and metabolites are released from the worms into culture supernatants. Transcripmtomic analysis shows that many Phase I and Phase II enzymes are up-regulated in *C. elegans* but not *H. contortus* when exposed to these benzimidazoles consistent with the glucosidation results. This work suggests that although benzimidazole biotransformation is biochemically similar in *C. elegans* and *H. contortus* it is less efficient, perhaps due reduced xenobiotic response efficiency, in the latter species.

2. Material and methods

2.1. Strains used

Using nematode strains containing mutations in the gene encoding the benzimidazole drug target, allows for acute anthelmintic exposures at very high concentrations to be used to induce xenobiotic responses without the worms being visibly adversely affected - having apparently normal movement and development. The *C. elegans* benzimidazole resistant strain CB3474 ben-1(c1880)III (Driscoll et al., 1989), was used for all drug exposure experiments. This strain is benomyyl resistant, and is dominant at 25 °C and recessive at 15 °C. The *H. contortus* UGA/2004 (Williamson et al., 2011), which is highly resistant to benzimidazoles, levamisole, and ivermectin, was to determine the transcriptional response to the benzimidazoles. The *H. contortus* MHco3 (ISE) (Otten et al., 2001; Roos et al., 2004) indbred genome reference strain, and MHco12 (von Samson-Himmelstjerna et al., 2009) a benzimidazole resistant strain were used to detect metabolites. Worms were vigorously motile after drug exposure times used.

*C. elegans* stock strains were maintained on NGM agar plates with OP50 *E. coli* as the food source as described (Brenner, 1974). The *C. elegans* benzimidazole resistant strain CB3474 ben-1(c1880)III was cultured in CER3 axenic culture (Nass and Hamza, 2007) to ensure that the metabolites produced were the product of worm metabolism and not the bacteria normally used as food source. Eggs were isolated and sterilized using sodium hypochlorite/sodium hydroxide, L1s were cultured in 175 cm2 tissue flasks (BD Falcon # 353112) with CER3 media as the sole food source, supplemented with Antibiotic-Antimyotic (Gibco # 15240062), and incubated at 20 °C with 50 tilts per minute rocking, until a density of > 10 worms/mL was achieved. TriPLICATE axenic cultures, of greater than 2.5 × 105 worms/mL were centrifuged at 3000xg for 5 min to pellet the
worms. The supernatant was removed and snap frozen at −80 °C. The worm pellet was washed with 40 mL of M9 solution and centrifuged at 3000xg for 15 min. The wash supernatant was removed from the worm pellet and both were snap frozen at −80 °C. Samples were kept at −80 °C until used to prepare samples for HPLC and LC-MS/MS.

2.3. Culturing of *H. contortus* and anthelmintic exposure for detection and characterization of metabolites

The *H. contortus* reference strain MHco3(ISE) was used to detect metabolites produced upon exposure to benzimidazole anthelmintics. Two sheep were orally infected with a dose of 5000 third stage infective larvae. Twenty-eight days post-infection, the animals were euthanized and sheep abomasae were surgically removed, all experimental procedures described here were approved by the Morehead Research Institute Experiments and Ethics Committee and were conducted under the legislation of a UK County Home Office License (reference PPL 60/03899) in accordance with the Animals (Scientific Procedures) Act of 1986. During worm counting and manipulation the *H. contortus* adults were held in pre-incubation media of RPMI-1640 medium (pH6.8, 38 °C) containing 10X antibiotic-antimycotic ( Gibco # 15240062). Triplicate samples of 150 adult worms were incubated in 7.5 mL of incubation media consisting of RPMI-1640 (pH6.8), supplemented with 0.8% (w/vol) glucose, 10 mM HEPES, 1X antibiotic-antimycotic ( Gibco # 15240062) in 25 cm² flat bottomed tissue flasks (BD Falcon Cat# 353108). At time zero, the benzimidazole anthelmintics: albendazole (Sigma Cat# A673), mebendazole (Sigma Cat# M2523), thiabendazole (Sigma Cat#T8904), oxfendazole (Sigma Fluka 34176), fenbendazole (Sigma Cat# F5396) were added to a final concentration of 10 μM, and the flasks then incubated at 38 °C with humid atmosphere with 10% CO₂ for 16 h, DMSO was 0.1% in all treatments. After 16 h of culture, adult worms were manually removed from media, washed in PBS and snap frozen at −80 °C. The culture supernatants were snap frozen at −80 °C and kept at −80 °C until sample preparation for HPLC and LC-MS/MS.

2.4. Metabolite extraction from *C. elegans* and *H. contortus* culture supernatants

The *C. elegans* CeHR-3 culture supernatant was centrifuged at 4000 rpm (3400xg) on a Sorvall Legend RT for 10 min; 20 mL of the 50 mL *C. elegans* culture supernatant, or the entire 7.5 mL of *H. contortus* culture supernatant was filtered through a 0.22 μm filter (Pall Life Sciences Acrodisc 25 mm syringe filter) and applied to an activated and equilibrated C18 solid phase extraction column (SPE; Sep-Pak Plus C18 cartridge (Waters Cat. No. WAT020515); column was activated with 5 mL 100% acetonitrile and equilibrated with 10 mL dH₂O). The SPE column was washed with 5 mL dH₂O. Metabolites and parental drug were eluted in 5 mL 100% acetonitrile and diluted 5 fold in dH₂O (OmniSolv grade), collected in 5 mL 100% acetonitrile and equilibrated with 10 mL dH₂O. The supernatant was centrifuged at 3000xg for 15 min. The wash supernatant was removed from the worm pellet was washed with 40 mL of M9 solution and centrifuged at full speed in a microcentrifuge for 5 min). The supernatant was filtered using a 0.22 μm filter, and analyzed by HPLC and LC-MS/MS.

2.5. Metabolite extraction from pellet *C. elegans* and *H. contortus* cultures

The worm pellet of each culture, as well as the wash of each worm pellet, was thawed on ice. A fraction of the pellet wash was used to resuspend the respective worm pellet to a final volume of 10 mL and a 1 mL aliquot was removed for protein quantification and stored at −80 °C. The worm pellet and pellet wash were separated by centrifugation. Metabolites and parental drug extraction from the pellet wash was carried out as described for metabolite extraction from worm culture supernatant (using the entire pellet wash). The worm pellet was ground under liquid nitrogen and metabolites and parental drug were extracted in a total of 3 mL of methanol over two extraction steps.

Samples were vortexed, sonicated (1–2 min), and centrifuged to remove worm debris (2-step centrifugation: 1) 5 min at 4000 rpm in the Sorvall Legend RT centrifuge and 2) supernatant was transferred to a 2-mL microtube and centrifuged at full speed in a microcentrifuge for 5 min). The supernatant was filtered using a 0.22 μm filter, and analyzed by HPLC and LC-MS/MS.

2.6. HPLC and LC-MS/MS analysis

HPLC analysis of SPE flow-through collections and elutions, as well as worm pellet extractions, were performed on a Waters 2795 Separations Module coupled with a Waters 2996 Photodiode Array Detector (PDA). Compounds present in a 5 μL injection (10 μL injection of pellet extract) were separated by a Sunfire C18 column (Waters; 3.5 μm × 4.6 mm x 150 mm; coupled with a Waters Sunfire C18 guard column, 3.5 μm × 4.6 mm x 20 mm) and a linear gradient of 80:20% water+1% acetic acid:acetonitrile to 100% acetonitrile over 10 min with a flow rate of 1 mL min⁻¹. Compounds were detected by UV within a 200–400 nm range. Analysis was conducted using Waters Empower software. The first two elution fractions from the SPE for both culture supernatant and pellet wash extractions were combined and diluted 5 fold in dH₂O. Metabolite extracts of pellet were diluted 5 fold with dH₂O. Five μL was injected on to an Eclipse Plus C18 column (Agilent; 1.8 μM × 21 mm x 50 mm) using an Agilent 1200 series LC system (G1379B degasser, G1312B binary pump SL, G1367D HIP-ALS SL +, and G1316B TCC SL) together with an Agilent 6410 Triple Quad LC-MS/MS. The mobile phase and gradient was identical to the HPLC with a flow rate of 0.4 mL min⁻¹. Samples were initially run using MS2 scan (TIC) in positive mode with a fragmentor set to 80 V. Once metabolites were identified, product ion scan was performed using the following masses for each drug treatment: For product ion scan, the fragmentor was set to 150 V, with collision energy of 30eV. Analysis was conducted using MassHunter software (Agilent Technologies).

2.7. Incubation of *C. elegans* in the presence of UDP-glucuronosyltransferase inhibitor chrys

Chrys is an UGT inhibitor of human UGT2B7, UGT1A1, UGT1A6, and UGT1A9 (Walsky et al., 2012). Triplicate axenic cultures of greater than 1.5 x 10⁶ *C. elegans* benzimidazole drug resistant strain, CB3474 ben-1(e1880)III (Driscoll et al., 1989), were exposed to 56.6 μM albendazole for 3 days either with or without the UGT inhibitor, chrys (Sigma Cat# C80105) at 200 μM. The relative amounts of the albendazole-glucose metabolite, with and without chrys, were determined by HPLC by measuring the area under the 7.4min peak at 306 nm wavelength (previously determined to be the albendazole-glucose metabolite peak). These data were made relative to the total protein in culture replicates using a BCA protein assay (Sigma Cat# 1001-491004).

2.8. Preconditioned supernatant containing metabolized albendazole used to test changes in potency

In 24 well plates containing 400 μL of liquid NGM, Gentamycin and OP50 *E. coli* (0.75% w/vol) as food source, the *C. elegans* benzimidazole drug resistant strain, CB3474 ben-1(e1880)III (Driscoll et al., 1989), were exposed for 10 days at 20°C to a fold serial dose range from 64 μM to 62.5 nM plus DMSO negative control (this is four times the final drug doses used in motility bioassay). 100 μL of this pre-conditioned supernatant along with 300 μL fresh NGM, containing Gentamycin, and OP50 *E. coli* (0.75% w/vol) and first larval stage (L1) of *C. elegans* N2 wild-type worms at final concentration of 1.5 L1/μL were aliquoted into each well, to achieve the standard drug curve range (16 μM–15.625 nM plus DMSO Negative control). To ensure that ten days incubation at 20°C does not decrease the potency of albendazole, an additional control of four times the standard drug dose of...
albendazole in culture medium without worms for 10 days was used, which was then diluted to the standard drug curve range and used in the bioassay. To ensure that albendazole was not sticking to the cuticle of the worms during the pre-incubation step, we exposed dead worms to four times the standard drug dose overnight. A control of fresh albendazole exposed to *C. elegans* N2 was used as comparison.

2.9. Digital analysis of *C. elegans* motility bioassay in response to albendazole anthelmintic exposure

Worm motility was used as a measure of drug potency. First stage larvae at a density of 1.5 L/μL were cultured in 400 μL in multiwell plate, in liquid NGM with OP50 *E. coli* as the food source along a standard drug curve range. A standard drug curve range for albendazole results in paralyzed worm at the higher drug doses and fully motile worms at the lower drug doses, this was previously found to be a 2-fold serial dilution of albendazole from 16 μM to 15.625 nM plus a negative DMSO control. Worms were cultured at 20 °C, rocking for 60 h to the early adult stage. After 60 h these culture plates were then placed in the Larval Motility Digital Analyzer (LaMDA), which uses a scanning digital camera to scan the wells, pixel data from sequential picture frames are taken every 300 ms, for 3 s and are assessed for changes in greyscale for every pixel coordinate in the field of view and converted to Mean Squared Error (MSE) (Lee, 1986; Du, 2013) as a measure of total movement and degree of paralysis of the worms. MSE values are calculated according to the equation: $\text{MSE} = \frac{1}{(i \cdot j)} \sum (a_{ij} - b_{ij})^2$, where $i$ and $j$ are the pixel coordinates and $a$ and $b$ are sequential picture frames. This gives a numerical value for total movement in a well, the MSE values were made relative to the DMSO negative control.

Three biological replicates in triplicate technical replicates were analyzed by non-linear regression analysis of the drug dose curves, using GraphPad Prism version 6.01, 4 parameter non-linear regression with top and base constrained. For one of the biological replicates, the 0.25 μM dose of ABZ exposed to dead worms did not receive the drug and this data point was removed from the analysis. Extra sum of square F test was used to analyse whether the logEC50 of fresh albendazole versus albendazole pre-exposed to live worms was significantly different at (p < 0.0001) in each of the biological replicates tested when the albendazole is pre-exposed to worms.

2.10. Transcriptional response to benzimidazole drug exposures

2.10.1. *C. elegans* culturing and drug exposure

*C. elegans* stock strains were maintained on NGM agar plates with OP50 *E. coli* as the food source as described (Brenner, 1974). Three biological replicates of approximately 20,000 L1 larvae *C. elegans* CB3474 ben-1(e1880)III were cultured in NGM media with *E. coli* OP50 at 1% (w/vol) at 20 °C for approximately 70 h until the adult stage, concentrations were kept consistent with micro-array experiment performed previously (Laing et al., 2010). Cultures were exposed to 1.13 mM of either albendazole (Sigma Cat# A4673), mebendazole (Sigma Cat# M2523), thiaabendazole (Sigma Cat# T8904), oxfendazole (Sigma Cat# 34176), fenbendazole (Sigma Cat# F5396) were added to a final concentration of 1.13 mM, and the flasks then incubated at 38 °C in humid atmosphere with 10% CO2 for 4 h. Worms were then picked from culture into Eppendorf tubes and stored in TRizol (%2011) was used to determine the transcriptional response to benzimidazole exposures, 2.9.1. Transcriptional response to benzimidazole drug exposures

2.10.2. Adult stage of *H. contortus* culturing and drug exposure

The triple resistant *H. contortus* UGA/2004 strain (Williamson et al., 2011) was used to determine the transcriptional response to benzimidazole exposure. Two sheep per strain were orally infected with a dose of 5000 third stage infective larvae. Twenty-eight days post-infection, the animals were euthanized, sheep abomasum were surgically removed, and the adult *H. contortus* collected. All animal experimental procedures described here were carried out in accordance with Agriculture & Agrifood Canada (AAFC, Lethbridge, AB, Canada) and Animal Care Committee (ACC) protocol #1613.

During worm counting and manipulation the *H. contortus* adults were held in pre-incubation medium (pH6.8, 38 °C) containing 10X antibiotic-antimycotic (Gibco # 15240062). Triplicate samples of 50 adult worms were incubated in 7.5 mL of incubation medium consisting of RPMI-1640 (pH6.8), supplemented with 0.8% (w/vol) glucose, 10mM HEPES, 1X antibiotic-antimycotic (Gibco # 15240062) in 25 cm2 flat bottomed tissue flasks (BD Falcon Cat # 353108). At time zero, worms were exposed to either DMSO solvent control or albendazole (Sigma Cat# A4673), mebendazole (Sigma Cat# M2523), thiaabendazole (Sigma Cat# T8904), oxfendazole (Sigma Fluka S4176), fenbendazole (Sigma Cat# F5396) were added to a final concentration of 1.13 mM, and the flasks then incubated at 38 °C in humid atmosphere with 10% CO2 for 4 h. Worms were then picked from culture into Eppendorf tubes and stored in TRizol (%2011) and immediately frozen at −80 °C until processing.

2.11. RNA extraction

RNA was extracted using TRizol as described by the manufacturer protocol (%2011); total RNA pellets were suspended in DEPC treated water. RNA was isolated and treated with DNaseI as per manufacturer protocol (Qiagen #74134). Only samples that had a minimum A260/A280 ratio of 1.7 and a RIN of greater than 8.0 were used. Total RNA extracted and sent to Genome Quebec [http://gqinnovationcenter.com] for template preparation and RNA-Seq Next Generation Sequencing on the Illumina HiSeq platform with PE100 to read depth of > 50,000,000 aligned reads.

2.12. RNA-seq analysis

Transcripts were quantified using Kallisto 0.46.0 (Bray et al., 2016) (default settings) with the *Haemonchus contortus* (PRJEBS056, WBPS13) or *Caenorhabditis elegans* (PRJNA13758, WS271) transcript coding sequences (CDS). All statistical analysis was done with R 3.6.0. Gene level analysis was done by first summarizing the transcripts to the gene level using tximport version 1.12.3 (Soneson et al., 2015) and then detecting differentially expressed genes with DESeq2 version 1.24.0 (Love et al., 2014), with an adjusted p-value cutoff of 0.05 for *H. contortus* samples and 0.01 for *C. elegans* samples. A stricter cutoff was used for *C. elegans* due to the large number of differentially expressed genes. Principal component analysis was done using the “plotPCA” function from the DESeq2 package. Functional analysis (over-representation test) for the *H. contortus* larva samples was done with the clusterProfiler (Yu et al., 2012) R package version 3.12.0 using the GO annotations from the PRJEBS056 assembly.

The data discussed in this publication have been deposited in NCBI’s Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE130466 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE130466).
Fig. 1. Chromatograms are the result of product total ion current (TIC), or a multiple reaction monitoring (MRM), of albendazole (ABZ) parental drug and its metabolites identified in the supernatant of cultures of either *C. elegans* benzimidazole resistant allele CB3474 ben-1(e1880)III (shown in green) or adult *H. contortus* drug sensitive MHco3(ISE) allele (shown in orange). The first (top) chromatograms corresponds to the culture containing benzimidazole but no nematodes, the second chromatograms corresponds to the culture containing *C. elegans* or *H. contortus*, respectively, exposed to albendazole (ABZ), the third chromatogram corresponds to the control culture containing *C. elegans* or *H. contortus* but with no benzimidazole. *C. elegans* was exposed to 56.5 μM BZ for 3 days, and *H. contortus* was exposed to 10 μM BZ for 16 h respectively. A. TIC chromatogram of *C. elegans* exposed to albendazole (ABZ). B. MRM chromatogram of *H. contortus* exposed to albendazole (ABZ). TIC chromatogram is shown in an insert. C. Metabolites and their LC-MS/MS fragmentation patterns identified in supernatant of the ABZ-treated *C. elegans* cultures (as indicated by the numbered labels on the second trace of panels A and B). D. Metabolites and their LC-MS/MS fragmentation patterns identified in supernatant of the ABZ-treated adult *H. contortus* cultures. The fragmentation patterns were extracted from either a Total Ion Current (TIC) of ABZ-treated *C. elegans* culture supernatant, or multiple reaction monitoring (MRM) of *H. contortus* culture and resulted from a fragmentor setting of 150 V and a collision energy of 30eV. E. Two diagnostic daughter ions derived from the m/z 428 ion were m/z 234 and m/z 266 of the fragmentation data. F. Three proposed isoforms formed by N-linked sugar, the most likely structures of these three isomers. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article).
Fig. 2. Chromatograms are the result of product Total Ion Current (TIC) or multiple reaction monitoring (MRM) of mebendazole (MBZ) parental drug and its metabolites identified in the supernatant of cultures of *C. elegans* benzimidazole resistant allele CB3474 ben-1(e1880)III (shown in green) or of adult *H. contortus* drug sensitive MHco3(ISE) allele (shown in orange). The first (top) chromatogram corresponds to the culture containing benzimidazole but no nematodes, the second chromatogram corresponds to the culture containing either *C. elegans* or *H. contortus* respectively, exposed to the benzimidazole, and the third chromatogram corresponds to the control culture containing *C. elegans* or *H. contortus* but with no benzimidazole. *C. elegans* was exposed to 56.5 μM BZ for 3 days, and *H. contortus* was exposed to 10 μM BZ for 16 h respectively. A. TIC chromatogram of *C. elegans* exposed to mebendazole (MBZ), B. TIC chromatogram of *H. contortus* exposed mebendazole (MBZ), C. Multiple reaction monitoring (MRM) chromatogram of *C. elegans* exposed to mebendazole (MBZ), D. Multiple reaction monitoring (MRM) chromatogram of *H. contortus* exposed to mebendazole (MBZ). Metabolites and their LC-MS/MS fragmentation patterns identified in supernatant of the MBZ-treated *C. elegans* or adult *H. contortus* cultures. These fragmentation patterns were extracted from a Total Ion Current (TIC) or multiple reaction monitoring (MRM) and resulted from a fragmentor setting of 150 V and a collision energy of 30eV. E. Metabolite 3 and its LC-MS/MS fragmentation pattern. F. Metabolite 4b and its LC-MS/MS fragmentation pattern. Two diagnostic daughter ions derived from the m/z 428 ion were m/z 234 and m/z 266 of the fragmentation data. G. Proposed sequential reaction of reduction and O-glycosylation to the phenyl ketone moiety in MBZ. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article).
3. Results

3.1. Similar biotransformation products of benzimidazole drug family members are released into in vitro culture supernatants by C. elegans and H. contortus

We investigated the biotransformation and export of each of five major benzimidazole drug family members in C. elegans and H. contortus by assaying axenic in vitro culture supernatants of each species for the presence of both parental drug and metabolites using LC-MS/MS (Figs. 1–4, Table 1). In each experiment, a control culture containing drug but no worms, and a control containing worms but no drug were included to ensure any benzimidazole metabolites detected were the product of nematode biotransformation. In the case of C. elegans, triply axenic cultures (Nass and Hamza, 2007) of approximately 1.5 × 106 C. elegans CB3247 ben-1(e1880)III were exposed to benzimidazoles: albendazole (ABZ: 225 m/z), mebendazole (MBZ: 295 m/z), fenbendazole (FBZ: 299 m/z), thiabendazole (TBZ: 201 m/z), and oxfendazole (OxFBZ: 315 m/z) at 56.5 μM BZ for 3 days, and H. contortus was exposed to 10 μM BZ for 16 h respectively. A. TIC scan of C. elegans exposed to fenbendazole (FBZ), B. TIC scan of H. contortus exposed to fenbendazole (FBZ), C. An extracted-ion chromatogram (EIC) of C. elegans exposed to fenbendazole (FBZ), D. MRM scan of H. contortus exposed to fenbendazole (FBZ). Metabolites and their LC-MS/Ms fragmentation patterns identified in supernatant of the FBZ-treated C. elegans or adult H. contortus cultures. These fragmentation patterns were extracted from a Total Ion Current (TIC) or multiple reaction monitoring (MRM) and resulted from a fragmentor setting of 150 V and a collision energy of 30 eV. E. Metabolite 5b from C. elegans and its LC-MS/Ms fragmentation pattern, F. Metabolite 6 and its LC-MS/Ms fragmentation pattern, G. Metabolite 5b from H. contortus and its LC-MS/Ms fragmentation pattern, H. Metabolite 7 and its LC-MS/Ms fragmentation pattern. I. A schematic summary of FBZ oxidative biotransformation is shown. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article).

ABZ, [ABZ + O], was identified in trace amounts in both worms by a targeted ion search using m/z 282. Although the oxygenated ABZ was also found in the ABZ-control without worms, co-incubation of worms with ABZ increased the production of oxygenated ABZ by 4.5-fold in C. elegans and by 34-fold in H. contortus (Supplemental Figure 4A/B). It appears that H. contortus produces relatively more oxygenated ABZ than C. elegans because ABZ is not efficiently glycosylated in H. contortus, thus providing more substrate for oxygenation. Similar H. contortus metabolites have previously been reported (Cvlinik et al., 2008b; Laing et al., 2010) (ABZ with S = O) by a characteristic daughter ion m/z 208 (Supplemental Figure 3C; Supplemental Figure 4C). Recent work from Stuchlikova et al. used UHPLC/MS/MS and identified additional novel ABZ metabolites in H. contortus: ABZ-sulphone, two N-glycosides with a hydroxylated carbamate side chain, and two ABZSO-N-glycosides (Stuchlikova et al., 2018).

In FBZ exposure assays, two new major compounds were produced from both C. elegans and H. contortus (Fig. 2A/2B). The compound 2 showed a [M + H] ion of m/z 298 while the compound 3 showed a [M + H] ion at m/z 460, indicating a 2 mass increase for 2 and an additional 162 mass increase for 3. The MS/MS analysis of 3 showed a 32 neutral loss as shown in ABZ but also showed a 180 mass neutral loss. The latter is a typical MS/MS fragmenting pattern for O-linked glycosylation on non-aromatic carbon (Fig. 2E) and the mechanism is shown in Supplemental Figure 5A. To explain the observed data, we propose the reaction scheme shown in Fig. 2G after considering structural differences between ABZ and MBZ. As the phenyl ketone group distinguishes MBZ from ABZ, the reduction and glycosylation should occur in the phenyl ketone group of MBZ. In this transformation, a reduction of the MBZ phenyl ketone group firstly occurred, followed by a hexose sugar conjugation to the newly formed hydroxyl group. Additional to these compounds, the new compounds with the MS/MS pattern (32 and 162 neutral losses) identical to that of the sugar-conjugated ABZ were also detected by MRM (Fig. 2C/2D/2F peaks 4a–4c), but these compounds (MBZ + hexose) were present significantly lower (~1%) than the compound 2 and 3 (MBZ + 2H and MBZ + 2H + hexose). These results demonstrated that a sequential reaction of reduction and O-glycosylation is a dominant metabolic route for MBZ in both C. elegans and H. contortus. This reaction is due to the unique phenyl ketone moiety in MBZ.

In FBZ exposure assays, the hexose sugar-conjugated products with 32 and 162 neutral losses in MS/MS were found in C. elegans (Fig. 3A/3E peaks 5a–5c) and were also detected in H. contortus only by MRM (Fig. 3D/3G peaks 5b and 5c). These are the compounds analogous to 1a-1c when ABZ was exposed to C. elegans. A second species of sugar-conjugated FBZ (Fig. 3A/3B peak 6) was detected in both C. elegans and H. contortus, but their MS/MS patterns do not match to the known MS/MS patterns for sugar-conjugated compound (i.e., 162 or 180 mass loss; Fig. 3F). Another type of sugar-conjugated FBZ was detected with typical 32 and 162 mass losses (Fig. 3C/3H peaks 7a-7c) only in C. elegans, but they showed a 16 mass increase in addition to a sugar conjugation. This mass increase suggested an oxygen addition as well as a hexose sugar, indicating that the detected molecule is a [FBZ + O + hexose + H]^+ ion. As the phenyl sulfide group is unique in FBZ, we
Fig. 4. Chromatograms are the result of product Total Ion Current (TIC) of thiabendazole (TBZ) or oxfendazole (OxBZ) parental drug and its metabolites identified in the supernatant of cultures of C. elegans benzimidazole resistant allele CB3474 ben-1(e1880)III (shown in green) or of adult H. contortus drug sensitive MHco3(ISE) allele (shown in orange). The first (top) chromatogram corresponds to the culture containing benzimidazole but no nematodes. The second chromatogram corresponds to the culture containing either C. elegans or H. contortus respectively, exposed to the benzimidazole. The third chromatogram corresponds to the control culture containing C. elegans or H. contortus but with no benzimidazole. C. elegans was exposed to 56.5 μM BZ for 3 days, and H. contortus was exposed to 10 μM BZ for 16 h respectively. LC-MS/MS fragmentation patterns identified in supernatant of the TBZ-treated C. elegans or adult H. contortus cultures. These fragmentation patterns were extracted from a Total Ion Current (TIC) and resulted from a fragmentor setting of 150 V and a collision energy of 30eV. A. TIC scan of C. elegans exposed to thiabendazole (TBZ), B. TIC scan of H. contortus exposed to thiabendazole (TBZ), C. Metabolite 8 and its LC-MS/MS fragmentation pattern, D. TIC scan of C. elegans exposed to oxfendazole (OxBZ), E. TIC scan of H. contortus exposed to oxfendazole, F. Proposed structure of metabolite 8. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article).

Table 1

Summary of metabolites produced by C. elegans CB3474 ben-1(e1880)III and H. contortus drug sensitive MHco3(ISE) as determined by LC-MS/MS analysis shown in Figs. 1–4. C. elegans were cultured with each benzimidazole drug at 56.5 μM for 3 days in axenic culture. H. contortus adult worms were cultured in RPMI-1640 plus supplements with 10 μM of each benzimidazole for 16 h at 38°C (10% CO2).

| C. elegans metabolites | H. contortus metabolites |
|------------------------|-------------------------|
| Parental | Primary product | Secondary product | Tertiary product | Primary product | Secondary product | Tertiary product |
| ABZ | ABZ+hexose | ABZ+O | MBZ+hexose | ABZ+O | ABZ+hexose | |
| MBZ | MBZ+hexose+2H | MBZ+2H | MBZ+hexose+2H | MBZ+2H | MBZ+hexose | |
| FRZ | FRZ+hexose | FRZ+O+hexose+H | Not found | FRZ+hexose | Not found | |
| TBZ | TBZ+O+hexose+H | Not found | Not found | TBZ+O+hexose+H | Not found | |
| OxBZ | OxBZ+hexose+H | OxBZ+hexose+H | OxBZ+hexose+H | OxBZ+hexose+H | OxBZ+hexose+H | |

* Trace amounts.
considered the formation of phenyl sulfoxide (phenyl S=O) to produce OxBZ from FBZ, but OxBZ could not be detected in FBZ exposed C. elegans, indicating that an oxygen atom was not added to sulfur but likely to be added to the phenyl ring. These data suggested that the major metabolized species for FBZ in C. elegans are N-linked glycosylated products (5a-5c) similar to those from ABZ. The compounds 6 and 7a-7c are minor products, and their chemical identities remain unknown.

In TBZ exposure assays, a new compound 8 with [M+H]+ ion at m/z 380 was detected in C. elegans (Fig. 4A), whereas no biotransformation of TBZ was detected in H. contortus (Fig. 4B). The mass increase of 178 from TBZ, together with the MS/MS pattern (Fig. 4C), suggested that an oxygen (16 mass) and a hexose sugar (162 mass) were added to TBZ to form [TBZ+O+hexose+H]+ ion. TBZ derivatives attached with a hexose sugar alone (i.e., [M+hexose+H]+ ion) could not be detected. Therefore, different from ABZ, MBZ, and FBZ, nitrogen atoms in TBZ are not glycosylated, and an oxygen addition to TBZ is required for the next hexose conjugation. The neutral loss of 162 mass indicated that the sugar is conjugated to the oxygen attached to the aromatic ring, and it mechanism is shown in (Supplemental Figure 5B). Taken together, a possible structure for 8 is shown in Fig. 4F. The same TBZ bio-transformation pattern was recently reported (Jones et al., 2015).

When OxBZ was exposed to either C. elegans or H. contortus, no major compound metabolized from OxBZ were initially detected in both worms (Fig. 4D/4E), indicating OxBZ is not efficiently metabolized in worms. Further targeted ion searches for glycosylated OxBZ (M + hexose + H; m/z = 478) identified trace amounts of glycosylated OxBZ in both worms (Supplemental Figure 6A/B). However, as was the case shown in FBZ (Fig. 3F), the MS/MS pattern of this compound does not match to typical MS/MS patterns of sugar-conjugated compound (i.e., 162 or 180 mass loss; Supplemental Figure 6C) and it structure remains unknown.

We examined whether the glycosylated drugs accumulate inside worms after grinding C. elegans in methanol. LC-MS analysis showed that the worm extracts accumulate the glycosylated drugs from five drugs less than 5% of those found in medium. We concluded that glycosylated drugs are efficiently secreted to medium.

A summary of the described metabolites of C. elegans and H. contortus are presented in Table 1 and highlights several things of interest. Firstly, similar metabolites are produced by these two nematodes, with two exceptions (i.e.: FBZ + hexose + O and TBZ + O + hexose + H), where these metabolites were found in C. elegans in trace amounts only. Secondly, as illustrated from Table 1, biotransformation of the BZs with a hexose or a hexose plus other moiety is a common reaction for all the drugs tested. A BZ drug resistant strain of H. contortus (MHco12) (von Samson-Himmelstjerna et al., 2009) was compared to the drug sensitive ISE strain to determine whether there were any differences in the amount of any of the metabolites produced. No significant differences were found and so this was not investigated further (data not shown).

3.2. Exposure of albendazole to live C. elegans worms decreases the potency of the anthelmintic

We tested whether the anthelmintic albendazole is modified to a less potent product by pre-exposing the anthelmintic to the benzimidazole resistant strain C. elegans CB3474 ben-1(e1880)III for 10 days and then testing the pre-conditioned supernatant which contains the metabolized albendazole, alongside several controls, in a motility bio-assay on wild type N2 C. elegans. To ensure that albendazole does not lose potency after ten days incubation at 20 °C, we included a control which contained albendazole in culture medium without worms for ten days. Another control, to ensure that albendazole was modified by the worms rather than removed from the experiment due to absorption to the worm’s cuticle during the pre-incubation step, involved albendazole exposed to dead worms. Finally, fresh albendazole exposed to C. elegans N2 was used as a negative control and as comparison. In all three bioreplicates the dose response curve was shifted to the right for the medium that had been pre-exposed to the C. elegans cultures relative to the various controls, suggesting that C. elegans modifies the albendazole to a less potent product (Fig. 5).

3.3. The UDP-glucuronosyltransferase inhibitor chrysin reduces albendazole biotransformation by C. elegans in culture

A toxicity assay was performed with several pharmacological inhibitors- piperonyl butoxide (a CYP inhibitor), T-cinnaminic acid (a GST inhibitor), verapamil (a PGP inhibitor), and chrysin (a UGT inhibitor) - to determine the highest dose that did not affect C. elegans development or brood size. Doses of 100 μM, 200 μM, 100 μM, 200 μM respectively, did not produce a toxic effect on worms (data not shown). The effect on the abundance of metabolite produced on single biological replicates with each of these inhibitors was then performed using LC LC-MS/MS; these results suggested that only chrysin (UGT inhibitor) caused a decrease in the amount of metabolite produced (data not shown). Therefore, the effect of chrysin on metabolite production was chosen for further analysis.
Fig. 6. Reduction of albendazole-glucose metabolite production in the presence of the UDP-glucuronosyltransferase inhibitor, chrysin. Triplicate samples of *C. elegans* CB3474 ben-1(e1880)III in axenic culture exposed to albendazole at 56.5 μM with and without the UGT inhibitor chrysin (200 μM) for 3 days, HPLC was performed on 20 mL worm supernatant, area under the metabolite peak (at 7.4 min, 306 nm) was measured and normalized to total protein in sample using BCA assay. The error bars represent ± SEM of three biological reps. ** represents a significant difference at, p < 0.005, Student T test.

Approximately 1.5 × 10^6 *C. elegans* CB3474 ben-1(e1880)III were axenically cultured, in triplicate, with 56.5 μM of albendazole either with or without 200 μM chrysin for 72 h. A 20 mL of culture supernatant was extracted and examined by HPLC and the area under the metabolite peak corresponding to the ABZ + hexose metabolite was measured for each sample. The absorbance values were then divided by the total amount of worm protein extracted from the worm pellet (BCA protein assay) in each culture to normalize for the total number of worms. The mean peak intensity of the 306 nm absorbance peak corresponding to the ABZ + hexose metabolite was significantly less, for the chrysin containing cultures compared to the control cultures (2.19 fold reduction, P = 0.0042) (Fig. 6).

3.4. Comparison of the transcriptional response of *C. elegans* to different members of the benzimidazole drug family

We compared the transcriptional response of *C. elegans* to five different benzimidazole drug family members, at drug exposure concentrations as used previously (Laing et al., 2010), in order to identify both shared and distinct features of the transcriptional response. Three biological replicates of approximately 20,000 L1 larvae *C. elegans* CB3474 ben-1(e1880)III were cultured in liquid media, with E. coli OP50 at 1% (w/vol) at 20°C for approximately 70 h until worms reached the adult stage. Worms were then exposed to 1.13 mM of either: ABZ, MBZ, TBZ, FBZ or OxBZ as well as a negative control solution of DMSO for 4 h.

Relatively few genes are up regulated > 2-fold (adjusted p value < 0.01) in response to exposure to each of oxfendazole (506 genes), thiabendazole (304 genes), albendazole (193 genes) and mebendazole (148 genes) (Fig. 7A). There was a common subset of 41 genes that were up regulated (> 2-fold, adjusted p value < 0.01) in response to exposure to all of the five benzimidazoles (listed in Table 2). Over half of these were genes encoding enzymes known to have a role in the biotransformation of small xenobiotic molecules including: 10 cytochrome P450s; 8 UDP-glycosyltransferases; and 2 glutathione-S-transferases; as well as oxidoreductases, dehydrogenases and reductases (Table 2). In addition, one nuclear hormone receptor (nhr-142), and several other genes (cdr-1, irg-2, and vem-1) known to be induced by other xenobiotics or bacterial infections were up regulated by each of the five benzimidazoles in all cases (Liao et al., 2002; Runko and Kapieljan, 2004; Wong et al., 2007; Estes et al., 2010). Hence, the core transcriptomic response of the *C. elegans* CB3474 ben-1(e1880)III strain, in terms of genes up-regulated for four of the benzimidazole family members, is overwhelmingly dominated by classical xenobiotic response genes. In contrast, fenbendazole exposure resulted in the transcriptional up-regulation of the greatest number of genes by far; a total of 2262 were up regulated > 2-fold relative to the DMSO control (adjusted p value < 0.01) with 1963 of these genes being specific to fenbendazole and not up regulated in response to the other benzimidazoles (Fig. 7A).

Complete Gene Ontology (GO) analysis was performed on the genes up-regulated > 2-fold in response to exposure by each of the benzimidazole drug using Panther (Mi et al., 2013). A summary of top hits (based upon a greater than 4.5 fold enrichment and p values less than 1.0e-4) of the Panther Over-representation Test (release 20190711) (Mi et al., 2017) of GO-Slim biological processes are summarized in Table 3. The Panther Over-representation Test found an over representation of the response to xenobiotic stimulus (GO:0009410) for all anthelmintics tested, and response to drug (GO:0042493), organic acid metabolic process (GO:0006082), cellular catalytic process (GO:0044248) and drug metabolic process (GO:0017144) for albendazole, mebendazole, thiabendazole, and oxfendazole, again consistent with a classical xenobiastic response (Table 3).

In the case of fenbendazole, for which a much greater number of unique genes were up-regulated, there was an enrichment of intercellular transport (GO:0042073), chemical and trans-synaptic transmission (GO:0099177, GO:0050804), cell surface, glutamate receptor and cAMP-mediated signaling pathways (GO:1905114, GO:0007215, GO:0019933, GO:0023051), and cilium organization (GO:0044782) (Table 3) suggesting secondary targets. This was investigated further, exposure of *C. elegans* CB3474 ben-1(e1880)III to 1.13 mM FBZ throughout development and resulted in a reduction in brood size and larval development that was not observed in ABZ exposed worms (Supplemental Figs. 8A and B); phenotype enrichment analysis (Angeles-Albores et al., 2016) showed chemosensory behavior variant (WBPPhenotype:0001049) and various movement variants (WBPPhenotype:0002295, WBPPhenotype:0002347, WBPPhenotype:0002300, WBPPhenotype:0000238), as well as male mating efficiency reduced phenotype (WBPPhenotype:0008434) and foraging reduced phenotype (WBPPhenotype:0000238) (Supplemental Fig. 8C).

Although the major focus of this work was to determine which biotransformation enzymes may be transcriptionally up-regulated to the benzimidazole drugs, we also mined the data for those genes down-regulated > 2 fold in response to drug exposure (Fig. 7B). Of the five drugs, oxfendazole exposure resulted in the greatest number of down regulated genes (total of 1728 genes), closely followed by fenbendazole exposure (total of 1695 genes). A high proportion of these genes were down-regulated in response to both drugs (a total of 759 shared down regulated genes). There were fewer genes down regulated in response to exposure to the other drugs; thiabendazole (225 genes), albendazole (50 genes), and mebendazole (20 genes). There were only four genes down-regulated in response to all the drugs (pad-3, bath-26, cest-1 and C30G12.2), bath-26 has been shown to be regulated by several xenobiotics (Rudgalvyte et al., 2013). As fenbendazole and oxfendazole had the greatest number of genes down-regulated Complete Gene Ontology (GO) analysis was performed on the genes down-regulated > 2-fold in response to these two benzimidazoles using Panther Overrepresentation Test of biological processes (Mi et al., 2017) (Supplementary Table 1). Transmembrane tyrosine kinase signaling pathway (GO:0007169), very long-chain fatty acid metabolic process (GO:0000038), protein phosphorylation and autophosphorylation (GO:0006468, GO:0046777), had the highest fold enrichment, again suggesting secondary targets.

3.5. Comparison of the *H. contortus* transcriptional response following exposure to different members of the benzimidazole drug family

TriPLICATE ex-vivo cultures of fifty *H. contortus* UGA/2004 (Williamson et al., 2011) adult worms, collected fresh from the sheep abomasum, were exposed to 1.13 mM of ABZ, MBZ, TBZ, FBZ, OxBZ or DMSO solvent control for 4 h. RNA was extracted and RNAseq analysis
performed using the same approaches as the *C. elegans* experiments. No genes were identified as expressed > 2-fold with an adjusted p-value of > 0.05 between any of the drug exposed populations and the DMSO control. Consequently, the experiment was repeated for three of these drugs - albendazole, fenbendazole and oxfendazole – but at a higher dose (3 mM). Principal component analysis (PCA plot) to visualize the variation in the samples found little clustering of treatment replicates (Supplemental Figure 7B) signifying the majority of variance was not due to drug treatment. Few genes were significantly differentially expressed at any fold change (at adjusted p < 0.05) with albendazole having the greatest number of differentially expressed genes (357 genes). However, none of these were differentially expressed at > 2 fold. Fenbendazole had no significantly differentially regulated genes, and oxfendazole had five genes which were differentially regulated, only 1 of these HCON 00141910 was regulated > 2 fold (results not shown).

Quadruplicate samples of exsheathed infective larval stage of the triple-resistant field isolate UGA/2004 *H. contortus* were exposed to either 1.13 mM albendazole or DMSO solvent control for 4 h. The PCA plot for the infective larval RNA-Seq did indicate that the majority (up to 88%) of the variance in these samples is due to the differences between drug treatments with the exception of single outliers (Supplemental Figure 7C). A total of 2920 transcripts were significantly differentially up-regulated (adj P < 0.05), and of these 226 genes were up regulated > 2-fold (Supplemental Table 2). The genes up-regulated > 2-fold did not include any classical xenobiotic response genes; however examination of all differentially expressed genes indicated that several classical xenobiotic response genes were up-regulated, albeit less than 2-fold, in contrast no xenobiotic response genes were found to be down-regulated (Supplemental Table 3). These included 2 genes with protein motif homology to short chain dehydrogenases, 8 genes with protein motif homology to cytochrome P450s, 2 genes with glutathione S-transferase domain containing protein motifs, and 6 genes with UDP-glucuronosyl UDP-glucosyltransferase domain containing protein motifs (Supplemental Table 3). Functional analysis of the differentially expressed genes performed with the clusterProfiler R package version 3.12.0 using the GO annotations from the PRJEBS06 assembly showed a top hit of oxidation-reduction processes (GO:00055114). A total of 2537 *H. contortus* infective larvae genes were significantly (at adj P < 0.05) down regulated when exposed to albendazole, of which 509 genes were differentially down regulated greater than 2 fold (Supplemental Table 2).

4. Discussion

4.1. Glucose conjugation is the predominant biotransformation pathway for multiple benzimidazole drugs in both *C. elegans* and *H. contortus*

We, and others, have previously shown that the predominant biotransformation reaction of albendazole in *C. elegans* and *H. contortus* is the conjugation of glucose to the parental molecule (Cvilink et al., 2008b; Laing et al., 2010; Vokral et al., 2013; Stuchlikova et al., 2018). In this work we demonstrate that hexose conjugation (mostly likely glucose) is also the predominant biotransformation reaction for four of the most therapeutically important benzimidazole drugs but with some differences (Table 1). We found that some of the metabolites were also reduced or oxidized (ABZ+O, MBZ+hexose+2H, FBZ+O+hexose +H, TBZ+O+hexose +H, or OxBZ+hexose +H) and several less abundant non-glucosidated products were also produced to varying degrees for each compound. In future experiments, different time courses or advancements in MS/MS technologies may elucidate more of the trace metabolites and provide further insight into metabolism of the benzimidazoles.

The observation that glucose conjugation is the predominant pathway for benzimidazole metabolism in these two nematode species is different to mammals where, to our knowledge, hexose conjugation of a benzimidazole drug has not been reported to date. In liver microsomes from deer, cattle, pigs, and sheep (Velik et al., 2005), albendazole is quickly metabolized to a sulfoxide derivative (ABZ-SO), the active form of the drug, and then slowly oxidized to albendazole sulfone. In the kidney, it was found that cattle, sheep, rats and mice metabolized albendazole to various albendazole sulfoxides and sulfones found in the urine (Gyurik et al., 1981). Our results for the benzimidazoles add to the growing evidence that hexose conjugation (particularly glucose) is a common biotransformation reaction of small xenobiotic compounds in nematodes. This contrasts with the situation in mammals where the major conjugation reaction of small xenobiotic compounds is either oxidation/reduction (Ding and Kaminsky, 2003; Gueguen et al., 2006), or the addition of glucuronic acid (Meech et al., 2012; Rowland et al., 2013). There is a growing list of examples where sugar conjugation of
Table 2
Sub-set of genes significantly (adjusted p values < 0.01) up-regulated greater than 2 fold, followed by their log2 fold change for C. elegans CB3474 ben-1(e1880)III exposed to 1.13 mM BZ for 4 h in response to all benzimidazoles tested.

| Gene ID | Putative enzyme | Gene description | ABZ Δ log2 fold | FBZ Δ log2 fold | MBZ Δ log2 fold | OxBZ Δ log2 fold | TBZ Δ log2 fold |
|---------|----------------|------------------|----------------|----------------|----------------|----------------|----------------|
| ah-5    | Phase I        | Aldehyde dehydrogenase | 2.25            | 1.64            | 1.25            | 1.28            | 1.74            |
| dn-23   | Phase I        | DeHydrogenases, Short chain | 3.28            | 3.76            | 2.82            | 8.20            | 3.90            |
| E02C12.10 | Phase I      | Uncharacterized oxidoreductase Dhs-27 | 7.20            | 12.83           | 6.38            | 15.47           | 8.33            |
| CS2A10.2 | Phase I        | Carboxylic ester hydrolase | 3.00            | 5.43            | 4.27            | 6.96            | 2.19            |
| F13H6.3 | Phase I        | Esterase CM06B1 | 2.35            | 2.15            | 2.74            | 4.07            | 1.27            |
| gqa-2   | Phase I        | Putative glucosylceramidase 2 | 4.28            | 6.95            | 3.34            | 7.60            | 5.53            |
| Y738C8.10 | Phase I     | FMN binding activity and oxidoreductase activity | 5.73            | 5.54            | 4.48            | 7.40            | 5.83            |
| T25G12.13 | Phase I     | ortholog of drx/b7 (dehydrogenase/reductase(SOR family) | 5.91            | 7.07            | 5.09            | 9.62            | 7.20            |
| T10B5.8 | Phase I        | predicted FMN binding and oxidoreductase activity | 2.96            | 1.95            | 1.62            | 5.33            | 4.16            |
| cyp-1A6 | Phase I | CYtochrome P450 family | 2.16            | 4.93            | 3.83            | 6.92            | 2.02            |
| cyp-1A4 | Phase I | CYtochrome P450 family | 2.26            | 3.03            | 3.47            | 8.14            | 1.78            |
| cyp-3A9 | Phase I        | CYtochrome P450 family | 1.60            | 3.05            | 3.94            | 3.81            | 1.36            |
| cyp-3A1 | Phase I | CYtochrome P450 family | 8.61            | 7.53            | 6.79            | 9.63            | 6.06            |
| cyp-3A2 | Phase I        | CYtochrome P450 family | 5.00            | 3.72            | 3.93            | 2.97            | 1.54            |
| cyp-3A3 | Phase I        | CYtochrome P450 family | 6.06            | 7.44            | 4.97            | 8.25            | 7.61            |
| cyp-3A4 | Phase I | CYtochrome P450 family | 5.26            | 5.63            | 5.46            | 6.52            | 5.88            |
| cyp-3A5 | Phase I | CYtochrome P450 family | 7.11            | 6.40            | 5.88            | 7.10            | 7.67            |
| cyp-3B2 | Phase I | CYtochrome P450 family | 4.37            | 4.96            | 2.91            | 7.04            | 3.80            |
| cyp-3C1 | Phase I | CYtochrome P450 family | 7.18            | 5.45            | 5.61            | 5.55            | 3.24            |
| ugt-8 | Phase II | UDP-GlucuronosylTransferase | 5.26            | 3.91            | 3.42            | 5.69            | 4.73            |
| ugt-9 | Phase II | UDP-GlucuronosylTransferase | 4.32            | 4.53            | 3.56            | 6.90            | 4.54            |
| ugt-14 | Phase II | UDP-GlucuronosylTransferase | 3.41            | 2.52            | 2.56            | 4.29            | 2.26            |
| ugt-29 | Phase II | UDP-GlucuronosylTransferase | 3.02            | 1.27            | 2.08            | 3.73            | 1.92            |
| ugt-33 | Phase II | UDP-GlucuronosylTransferase | 3.50            | 5.49            | 2.44            | 9.24            | 2.76            |
| ugt-34 | Phase II | UDP-GlucuronosylTransferase | 5.41            | 5.76            | 3.06            | 6.99            | 4.62            |
| ugt-37 | Phase II | UDP-GlucuronosylTransferase | 1.68            | 4.09            | 2.09            | 3.39            | 3.32            |
| ugt-41 | Phase II | UDP-GlucuronosylTransferase | 1.89            | 1.61            | 1.54            | 3.38            | 1.30            |
| dri-5 | Phase II | Probable glutathione-S-transferase 5 | 2.29            | 3.18            | 2.24            | 4.42            | 1.93            |
| dri-21 | Phase II | Glutathione-S-Transferase | 1.73            | 2.08            | 1.35            | 5.89            | 2.15            |
| C295T.2 | Phase II | Predicted transferase, transfers phosphorous-containing groups | 4.10            | 4.09            | 2.87            | 4.07            | 2.25            |

As the majority of metabolites produced in C. elegans were glucosidated, we tested the effect of inhibiting the UDP-glucuronosyltransferase enzymes with chrysin to determine the effects on metabolite production. There was a 2-fold reduction in the ABZ+hexose metabolite when cultured in the presence of chrysin (at 200 μM). Since, chrysin has a relatively high degree of specificity as a UGT inhibitor (Walsky et al., 2012), this result suggests that UDP-glucuronosyltransferase enzymes are likely to play a role in the production of this metabolite. We also found that the biotransformation of albendazole in culture supernatants by C. elegans reduces the potency of the drug (Fig. 5). This suggests that upregulation of the relevant biotransformation enzymes, likely the UGTs, could be protective and so play a role in benzimidazole resistance. There is growing evidence to support this hypothesis; two benzimidazole resistant H. contortus strains (WR and BR) were shown to have greater UDP-glucosyltransferases enzyme activity compared to a sensitive strain (ISE) (Vokral et al., 2013). Also, there was an up-regulation of UGT368B2 in two BZ resistant strains (IRB and WR) compared to the sensitive ISE strain (Matouskova et al., 2018). Further, three flubendazole resistant strains (WR, BR and ISE-S) produced more of the metabolites compared to the ISE sensitive strain (Vokral et al., 2012). In contrast, we did not detect a significant increase in the production of benzimidazole glucoside metabolites between the MHco12 benzimidazole resistant strain (von Samson-Himmelstjerna et al., 2009) compared to MHco3(ISE) (Redman et al., 2008) strain for any of the BZs tested in our experiments (data not shown). It is not clear whether this is due the parasite strains, drugs
The dominant xenobiotic transcriptional response to benzimidazole in *C. elegans* is more muted in *H. contortus* with stage-specific differences

We examined the transcriptomic response in *C. elegans* to five different benzimidazoles to determine whether each benzimidazole induces its own specific xenobiotic response, or, whether there was a common benzimidazole xenobiotic response. The *C. elegans* benzimidazole resistant strain CB3474 *ben-1*(c1880)III, which carries a null mutation in the gene encoding the major β-tubulin drug target, was used to minimize stress responses and so enable us to focus on the xenobiotic response itself. Analysis of the Gene Ontology (GO) of complete biological processes (Mi et al., 2017) show that up-regulated genes to each of the benzimidazole drugs were dominated by xenobiotic metabolic response terms. A core set of 41 genes were up-regulated to all the benzimidazoles tested and over 50% (31 genes) of this core group were associated with classic Phase I and Phase II xenobiotic response genes based on their gene descriptions (Table 2). Given the nature of the benzimidazole biotransformation products in *C. elegans* the UDP-glucuronosyltransferase enzymes and the cytochrome P450 genes are of particular interest. Of the UDP-glucuronosyltransferase enzymes UGT-8, 9, 14, 25, 33, 34, 37, 41 would be priorities for further investigation.

### Table 3

Summary of Complete Gene Ontology (GO Slim) biological process analysis of the *C. elegans* CB3474 *ben-1*(c1880)III for differentially expressed genes, significantly up-regulated greater than 2-fold using Panther Overrepresentation Test (release 2019-0711) with FDR correction. Cut off for GO terms selected for this table: fold enrichment of > 4.5 fold.

| PANTHER GO-Slim Biological Process | C. elegans REFLIST (19921) | Input | Expected | over/under | Fold Enrichment | P-value |
|-----------------------------------|-----------------------------|-------|----------|------------|-----------------|---------|
| **ABZ**                           |                             |       |          |            |                 |         |
| response to xenobiotic stimulus    | 40                          | 12    | 0.39     | +          | 31.13           | 6.0E-14 |
| response to drug                   | 58                          | 12    | 0.56     | +          | 21.47           | 2.7E-12 |
| organic acid metabolic process     | 76                          | 12    | 0.73     | +          | 16.38           | 4.4E-11 |
| cellular catabolic process         | 91                          | 12    | 0.88     | +          | 13.68           | 2.9E-10 |
| drug metabolic process             | 91                          | 12    | 0.88     | +          | 13.68           | 2.9E-10 |
| innate immune response             | 120                         | 13    | 1.16     | +          | 11.24           | 4.8E-10 |
| cellular response to chemical      | 112                         | 12    | 1.08     | +          | 11.12           | 2.5E-09 |
| defense response                   | 123                         | 13    | 1.19     | +          | 10.97           | 6.3E-10 |
| response to stress                 | 212                         | 14    | 2.04     | +          | 6.85            | 3.8E-08 |
| response to chemical               | 187                         | 12    | 1.8      | +          | 6.66            | 4.9E-07 |
| **MBZ**                           |                             |       |          |            |                 |         |
| response to xenobiotic stimulus    | 40                          | 17    | 0.3      | +          | 57.21           | 2.4E-23 |
| response to drug                   | 58                          | 17    | 0.43     | +          | 39.45           | 4.5E-21 |
| organic acid metabolic process     | 76                          | 17    | 0.56     | +          | 30.11           | 2.4E-19 |
| cellular catabolic process         | 91                          | 17    | 0.68     | +          | 25.15           | 3.4E-18 |
| drug metabolic process             | 91                          | 17    | 0.68     | +          | 25.15           | 3.4E-18 |
| cellular response to chemical      | 112                         | 19    | 0.83     | +          | 22.83           | 1.6E-19 |
| defense response                   | 187                         | 20    | 1.39     | +          | 14.4            | 6.3E-17 |
| response to stimulus              | 627                         | 23    | 4.66     | +          | 4.94            | 4.4E-10 |
| **TBZ**                           |                             |       |          |            |                 |         |
| response to xenobiotic stimulus    | 40                          | 12    | 0.6      | +          | 19.92           | 1.1E-11 |
| response to drug                   | 58                          | 12    | 0.87     | +          | 13.74           | 4.5E-10 |
| organic acid metabolic process     | 76                          | 12    | 1.14     | +          | 10.48           | 6.9E-09 |
| cellular response to chemical      | 112                         | 16    | 1.69     | +          | 9.49            | 7.8E-11 |
| drug metabolic process             | 91                          | 12    | 1.37     | +          | 8.76            | 4.2E-08 |
| cellular response to chemical      | 120                         | 13    | 1.81     | +          | 7.19            | 9.5E-08 |
| defense response                   | 123                         | 13    | 1.85     | +          | 7.02            | 1.2E-07 |
| response to chemical               | 187                         | 19    | 2.82     | +          | 6.75            | 2.7E-10 |
| **OxBZ**                          |                             |       |          |            |                 |         |
| response to xenobiotic stimulus    | 40                          | 24    | 1        | +          | 24.1            | 1.1E-12 |
| response to drug                   | 58                          | 24    | 1.44     | +          | 16.62           | 9.1E-20 |
| organic acid metabolic process     | 76                          | 24    | 1.89     | +          | 12.68           | 1.5E-17 |
| drug metabolic process             | 91                          | 25    | 2.27     | +          | 11.03           | 5.0E-17 |
| cellular catabolic process         | 91                          | 24    | 2.27     | +          | 10.59           | 4.6E-16 |
| cellular response to chemical      | 112                         | 28    | 2.79     | +          | 10.04           | 5.3E-18 |
| defense response                   | 187                         | 29    | 4.66     | +          | 6.23            | 8.0E-14 |
| **FBZ**                           |                             |       |          |            |                 |         |
| intracellular transport            | 11                          | 11    | 1.23     | +          | 8.94            | 2.6E-06 |
| regulation of trans-synaptic       | 14                          | 11    | 1.57     | +          | 7.02            | 1.2E-05 |
| signaling                         | 14                          | 11    | 1.57     | +          | 7.02            | 1.2E-05 |
| modulation chemical synaptosomal   | 9                            | 7     | 1.91     | +          | 6.95            | 5.2E-04 |
| transmission signaling pathway     | 8                            | 6     | 0.9      | +          | 6.7             | 1.5E-03 |
| glutamate receptor signaling       | 15                          | 11    | 1.68     | +          | 6.55            | 1.9E-05 |
| pathway                           | 15                          | 11    | 1.68     | +          | 6.55            | 1.9E-05 |
| synaptic transmission, glutamateric| 30                          | 20    | 3.36     | +          | 5.96            | 2.5E-08 |
| cell organization                  | 29                          | 16    | 3.13     | +          | 5.11            | 2.8E-06 |
| protein localization to cilium     | 40                          | 22    | 4.48     | +          | 4.92            | 6.6E-08 |
| response to xenobiotic stimulus    | 40                          | 13    | 2.8      | +          | 4.65            | 3.1E-05 |

used, or other methodological differences but warrants further investigation.

4.3. The dominant xenobiotic transcriptional response to benzimidazole in *C. elegans* is more muted in *H. contortus* with stage-specific differences
validates our results, and suggests that these 43 genes are the key xenobiotic response group to benzimidazoles in *C. elegans*.

The transcriptional response of *C. elegans* CB3474 ben-1(c1880)III when exposed to fenbendazole showed many more uniquely differentially expressed genes, as well as reduced brood size and larval development, fenbendazole inhibition of ubiquitin-proteasome function and induction of endoplasmic reticulum stress in human cancer cells has been reported (Dogra and Mukhopadhyay, 2012), suggesting fenbendazole has secondary targets in addition to the well characterised β-tubulin target.

Albendazole, mebendazole and fenbendazole, and oxendazole metabolites similar to those found in *C. elegans* were also present in adult *H. contortus* cultures suggesting that similar xenobiotic biotransformation may occur in these nematodes. There were inevitable differences in the culture conditions and tissue mass between *C. elegans* and *H. contortus*, however, the results suggest the biotransformation processes are less efficient in adult *H. contortus* than in *C. elegans*. Consistent, with this hypothesis, the global transcriptomic response to benzimidazole drugs of adult *H. contortus* worms was less marked than that of *C. elegans* with no genes being significantly up-regulated > 2-fold (adjusted p-value > 0.05) and with no apparent enrichment of xenobiotic response genes in response to any of the five drugs. These experiments were conducted at the same (and also higher) drug concentrations, and same exposure times as for *C. elegans*. *H. contortus* was viable for the length of the *in vitro* culture as demonstrated by vigorous movement after the 4 h drug exposure. Nevertheless, one caveat is that the adult parasites are in a highly abnormal environment when *in vitro* culture and may not behave in a physiologically relevant manner. The investigation of transcriptional responses of *H. contortus* to benzimidazoles *in vivo* in the host would be of interest to test this possibility.

We also investigated the transcriptional response to albendazole exposure of *H. contortus* infective L3 to test for stage-specific differences in xenobiotic responses. Although *H. contortus* infective L3 had a less marked transcriptomic response than *C. elegans*, they did show some evidence of a muted xenobiotic response to albendazole exposure. It has been hypothesised that parasites in the host environment have less need for reactive xenobiotic responses than organisms living in a more dynamic free-living environment (Zarowiecki and Berriman, 2015).

Recent genomic analysis (review see (Zarowiecki and Berriman, 2015; Matouskova et al., 2016)) of several helminths has shown that the free living nematode *C. elegans* have many xenobiotic metabolizing genes (86 CYPs, 68 SDR, 48 GSTs, 72 UGTs and 60 ABC transporters) (Lindblom and Dodd, 2006). In contrast, parasitic platyhelminths, with life cycles less exposed to the environment, have very small numbers of drug metabolizing gene families (e.g. 1 or 2 CYPs each and no UGTs detected) (Berriman et al., 2009; Olson et al., 2012; Young et al., 2012; Cwiklinski et al., 2015). The parasitic nematodes are more variable; however, there is a trend that those parasites that do not have free-living stages (e.g. *Trichinella* and filarial parasites) have fewer drug metabolizing gene family members. In contrast, the strongyloid nematodes (which have free-living stages as part of their life cycle) tend to have larger xenobiotic metabolizing enzyme gene families (Zarowiecki and Berriman, 2015); *H. contortus* has 42 CYPs, 70 SDR, 28 GSTs, 34 UGTs and 46 ABC transporters in the current published draft genome assembly (Laing et al., 2013). The transcriptional profile of a panel of *H. contortus* cyp genes showed that 15 out of 19 tested were significantly down-regulated in the adult stage compared to the free-living L3 stage (Laing et al., 2015). Hence, an interesting hypothesis to test will be whether the regulatory elements of genes encoding xenobiotic response elements are less responsive to induction in the parasitic stages compared to the free-living stages of parasites such as *H. contortus*.

Conflicts of interest

The authors have no competing interests to declare.

Akkas, D., Yalinkilinc, H.S., Sekkin, S., Boyaciglu, M., Girak, V.Y., Ayaz, E., Gokbulut, C., 2015. Comparative pharmacokinetics and bioavailability of albendazole sulfoxide in sheep and goats, and dose-dependent plasma disposition in goats. BMC Vet. Res. 11, 124.

Albonico, M., Bickle, Q., Ramans, M., Montresor, A., SavioLi, T., 2003. Efficacy of mebendazole and levamisole alone or in combination against intestinal nematode infections after repeated targeted mebendazole treatment in Zanzibar. Bull. World Health Organ. 81, 343–352.

AlGushi, S., Krucken, J., Ramunke, S., von Samson-Himmelstjerna, G., Demeler, J., 2014. Analysis of putative inhibitors of anthelmintic resistance mechanisms in cattle gastrointestinal nematodes. Int. J. Parasitol. 44, 647–658.

Alvarez-Sanchez, M.A., Perez-Garcia, J., Cruz-Rojo, M.A., Rojo-Vazquez, F.A., 2005. Real time PCR for the diagnosis of benzimidazole resistance in trichostongylids of sheep. Vet. Parasitol. 129, 291–298.

Ameyaw, D.A., Napuran, R., To, T.C., Ranson, H., Spillings, B.L., Wood, O.R., Brooke, B.D., Coetzee, M., Koekemoer, L.L., 2008. Over expression of a cytochrome P450 (CYP6P9) in a major African malaria vector, Anopheles Funestus, resistant to pyrethroids. Insect Mol. Biol. 17, 19–25.

Angeles-Albores, D., By, N.L., Chan, J., Sternberg, P.W., 2016. Tissue enrichment analysis for *C. elegans* genomics. BMC Biol. 17, 366.

Berriman, M., Haas, B.J., LeVerde, P.T., Wilson, R.A., Dillon, G.P., Cerqueira, G.C., Madsen, S.T., Al-Lazikani, B., Andrade, L.F., Ashton, P.D., Aslett, M.A., Barnes, D.C., Blandin, G., Caffrey, C.R., Coghlan, A., Coulson, R., Delcher, A., DeMarco, R., Dijkeng, A., Eyre, T., Gamble, J.A., Gedin, E., Gu, Y., Hertz-Fowler, C., Hirai, H., Hirai, Y., Houston, R., Ivens, A., Johnston, D.A., Lacerda, D., Macedo, C.D., McVeigh, P., Ning, Z., Oliveira, G., Overington, J.P., Parkhill, J., Pertece, M., Pierce, R.J., Protasio, A.V., Quail, M.A., Rajendream, M.A., Rogers, J., Sajid, M., Salzberg, S.L., Stance, M., Tivey, A.R., White, O., Williams, D.L., Wortman, J., Wu, W., Zamanian, M., Zerlotini, A., Fraser-Liggett, C.M., Barrett, R.B., El-Sayed, M.N., 2009. The genome of the blood fluke Schistosoma mansoni. Nature 460, 352–358.

Bray, N.L., Fintzelt, H., Melsted, P., Pletcher, L., 2016. Near-optimal probabilistic RNA-seq quantification. Nat. Biotechnol. 34, 525–527.

Breimer, S., 1974. The genetics of Caenorhabditis elegans. Genetics 77, 71–94.

Burglin, T.R., Lobos, E., Blaxter, M.L., 1998. Caenorhabditis elegans as a model for parasitic nematodes. Int. J. Parasitol. 28, 395–411.

Burns, A.R., Luciani, G.M., Musso, G., Bugu, R., Yeo, M., Zhang, Y., Rajendran, L., Glavin, J., Hunter, R., Redman, E., Stasiuk, S., Schertzberg, M., Angus McQuibban, G., Caffrey, C.R., Cutler, S.R., Tyers, M., Giaever, G., Nislow, C., Fraser, A.G., MacRae, C.A., Gillette, J., Roy, P.J., 2015. Caenorhabditis elegans is a useful model for anthelmintic discovery. Nat. Commun. 6, 7485.

Burns, A.R., Wallace, I.M., Wildenhain, J., Tyers, M., Giaever, G., Ruder, G.D., Nislow, C., Cutler, S.R., Roy, P.J., 2010. A predictive model for drug bioaccumulation and bioactivity in Caenorhabditis elegans. Nat. Chem. Biol. 6, 549–557.

Civluk, V., Kubicek, V., Nolibis, M., Krizova, V., Sotakova, B., Lamka, J., Varady, M., Kohouova, M., Novotna, R., Gavelova, M., Slavoka, I., 2008a. Biotransformation of flubendazole and selected model xenobiotics in Haemonchus contortus. Vet. Parasitol. 151, 242–248.

Civluk, V., Sotakova, B., Sotakova, B., Lamka, J., Kostianin, R., Kolota, R.A., 2008b. LC-MS-MS identification of albendazole and flubendazole metabolites formed ex vivo by Haemonchus contortus. Anal. Bioanal. Chem. 391, 337–343.

Civluk, V., Sotakova, B., Krivaova, V., Lamka, J., Slavoka, I., 2009a. Phase I biotransformation of albendazole in lancel fluke (*Dicrocoelium dendriticum*). Res. Vet. Sci. 86, 49–55.

Civluk, V., Sotakova, B., Vokral, I., Bartlikova, H., Lamka, J., Slavoka, I., 2009b. Liquid chromatography/mass spectrometric identification of benzimidazole anthelmintics metabolites formed ex vivo by Dicrocoelium dendriticum. Rapid Commun. Mass Spectrom. : RCM (Rapid Commun. Mass Spectrom.) 23, 2679–2684.

Cwiklinski, K., Dulton, D.J.P., Dufresne, P.J., La Courte, J., Williams, D.J., Hodgkinson, J., Paterson, S., 2015. The Fasciola hepatica genome: gene duplication and polynorphism reveals adaptation to the host environment and the capacity for rapid evolution. Genome Biol. 16, 71.

Daborn, P.J., Yen, J.L., Bogwitz, M.R., Le Goff, G., Feil, E., Jeffers, S., Tijet, N., Perry, T., 2013.
Dimunova, D., Nguyen, L.T., Varady, M., Skalova, L., 2018. Metabolism of albendazole, flubendazole and flubendazole in Haemonchus contortus adults: sex differences, resistance-related differences and the identification of new metabolites. Int. J. Parasitol. Drugs Drug Resist. 8, 50–58.

Stupp, G.S., von Reuss, S.H., Izrayelit, Y., Ajredini, R., Schroeder, F.C., Edison, A.S., 2013. Chemical detoxification of small molecules by Caenorhabditis elegans. ACS Chem. Biol. 8, 309–313.

Velik, J., Baliharova, V., Skalova, L., Szotakova, B., Wsol, V., Lamka, J., 2005. Liver microsomal biotransformation of albendazole in deer, cattle, sheep and pig and some related wild breeds. J. Vet. Pharmacol. Ther. 28, 377–384.

Vokral, I., Bartikova, H., Prchal, L., Stuchlikova, B., Szotakova, B., Lamka, J., Varady, M., Kubicek, V., 2012. The metabolism of flubendazole and the activities of selected biotransformation enzymes in Haemonchus contortus strains susceptible and resistant to anthelmintics. Parasitology 139, 1309–1316.

Vokral, I., Jirasko, R., Stuchlikova, B., Bartikova, H., Szotakova, B., Lamka, J., Varady, M., Skalova, L., 2013. Biotransformation of flubendazole and activities of selected detoxification enzymes in Haemonchus contortus strains susceptible and resistant to anthelmintics. Vet. Parasitol. 196, 373–381.

von Samson-Himmelstjerna, G., Blackhall, W., 2005. Will technology provide solutions for drug resistance in veterinary helminths? Vet. Parasitol. 132, 223–239.

von Samson-Himmelstjerna, G., Walsh, T.K., Donnan, A.A., Carriere, S., Jackson, F., Skuce, P.J., Rohn, K., Wolstenholme, A.J., 2009. Molecular detection of benzimidazole resistance in Haemonchus contortus using real-time PCR and pyrosequencing. Parasitology 136, 349–358.

Walsky, R.L., Bauman, J.N., Bourcier, K., Giddens, G., Lapham, K., Nagabban, A., Ryder, T.F., Obach, R.S., Hyland, R., Gosen, T.C., 2012. Optimized assays for human UDP-glucuronosyltransferase (UGT) activities: altered alamethicin concentration and utility to screen for UGT inhibitors. Drug Metab. Dispos. 40, 1051–1065.

Ward, J.D., 2015. Rendering the intractable more tractable: tools from Caenorhabditis elegans ripe for import into parasitic nematodes. Genetics 201, 1279–1294.

Williamson, S.M., Storey, B., Howell, S., Harper, K.M., Kaplan, R.M., Wolstenholme, A.J., 2011. Candidate anthelmintic resistance-associated gene expression and sequence polymorphisms in a triple-resistant field isolate of Haemonchus contortus. Mol. Biochem. Parasitol. 180, 99–105.

Winterrowd, C.A., Pomroy, W.E., Sangster, N.C., Johnson, S.S., Geary, T.G., 2003. Benzimidazole-resistant beta-tubulin alleles in a population of parasitic nematodes (Cooperia oncophora) of cattle. Vet. Parasitol. 117, 161–172.

Wolstenholme, A.J., Fairweather, I., Prichard, R., von Samson-Himmelstjerna, G., Sangster, N.C., 2004. Drug resistance in veterinary helminths. Trends Parasitol. 20, 469–476.

Wong, D., Rozopoulos, D., Pujol, N., Tavernarakis, N., Ewbank, J.J., 2007. Genome-wide investigation reveals pathogen-specific and shared signatures in the response of Caenorhabditis elegans to infection. Genome Biol. 8, R194.

Young, N.D., Jex, A.R., Li, B., Liu, S., Yang, L., Xiong, Z., Li, Y., Cantacessi, C., Hall, R.S., Xu, X., Chen, F., Wu, X., Zerlotini, A., Oliveira, G., Hofmann, A., Zhang, G., Fang, X., Kang, Y., Campbell, B.E., Loukas, A., Ranganathan, S., Rollinson, D., Rinaldi, G., Brandley, P.J., Yang, H., Wang, J., Wang, J., Gasser, R.B., 2012. Whole-genome sequence of Schistosoma haematobium. Nat. Genet. 44, 221–225.

Yu, G., Wang, L.G., Han, Y., He, Q.Y., 2012. clusterProfiler: an R package for comparing biological themes among gene clusters. OMICS A J. Integr. Biol. 16, 284–287.

Zamanian, M., Cook, D.E., Lee, D., Lee, J., Andersen, E., 2017. Heritable Small RNAs Regulate Nematode Benzimidazole Resistance. bioRxiv.

Zamanian, M., Cook, D.E., Zdralejivc, S., Brady, S.C., Lee, D., Lee, J., Andersen, E.C., 2018. Discovery of genomic intervals that underlie nematode responses to benzimidazoles. PLoS Neglected Trop. Dis. 12, e0006368.

Zanger, U.M., Schwab, M., 2013. Cytochrome P450 enzymes in drug metabolism: regulation of gene expression, enzyme activities, and impact of genetic variation. Pharmacol. Ther. 138, 103–141.

Zarowiecki, M., Bertram, M., 2015. What helminth genomes have taught us about parasite evolution. Parasitology 142 (Suppl. 1), S85–S97.