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Metabolism of albendazole, ricobendazole and flubendazole in *Haemonchus contortus* adults: Sex differences, resistance-related differences and the identification of new metabolites

Lucie Raisová Stuchlíková[a], Petra Matoušková[a], Ivan Vokřál[b], Jiří Lamka[b], Barbora Szotáková[a], Anna Sečkařová[a], Diana Dimunová[a], Linh Thuy Nguyen[a], Marián Várady[c], Lenka Skálová[a,*]

[a] Department of Biochemical Sciences, Faculty of Pharmacy, Charles University, Heyrovského 1203, Hradec Králové, Czech Republic
[b] Department of Pharmacology and Toxicology, Faculty of Pharmacy, Charles University, Heyrovského 1203, Hradec Králové, Czech Republic
[c] Institute of Parasitology, Slovak Academy of Sciences, Hlinkova 3, Košice, Slovakia

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ABSTRACT

Haemonchus contortus (family *Trichostrongylidae, Nematoda)*, a hematophagous gastrointestinal parasite found in small ruminants, has a great ability to develop resistance to anthelmintic drugs. We studied the biotransformation of the three benzimidazole anthelmintics: albendazole (ABZ), ricobendazole (albendazole S-oxide; RCB) and flubendazole (FLU) in females and males of *H. contortus* in both a susceptible ISE strain and resistant IRE strain. The *ex vivo* cultivation of living nematodes in culture medium with or without the anthelmintics was used. Ultrasensitive UHPLC/MS/MS analysis revealed 9, 7 and 12 metabolites of ABZ, RCB and FLU, respectively, with most of these metabolites now described in the present study for the first time in *H. contortus*. The structure of certain metabolites shows the presence of biotransformation reactions not previously reported in nematodes. There were significant qualitative and semi-quantitative differences in the metabolites formed by male and female worms. In most cases, females metabolized drugs more extensively than males. Adults of the IRE strain were able to form many more metabolites of all the drugs than adults of the ISE strain. Some metabolites were even found only in adults of the IRE strain. These findings suggest that increased drug metabolism may play a role in resistance to benzimidazole drugs in *H. contortus*.

1. Introduction

Haemonchus contortus (family *Trichostrongylidae, Nematoda)* is a hematophagous gastrointestinal parasite found in small ruminants that causes substantial economic losses to livestock production worldwide (Kaminsky et al., 2008). *H. contortus* has a great ability to develop resistance to anthelmintics (Kotze and Prichard, 2016), with drug resistance having become a major obstacle which threatens farm production and the welfare of the animals. Among parasites, the recent emergence of drug resistance to currently available drugs has raised serious problems for the control strategies and eventual elimination of parasitic diseases (Choe et al., 2012; Taman and Azab, 2014; Srivastava and Misra-Bhattacharya, 2015). The development of variable degrees of resistance among nematodes has been reported for all groups of anthelmintic drugs and increased level of resistance should be expected due to the considerable rise in drug administration, which increased drug pressure toward the selection of resistance alleles (Geary, 2012). The fact that resistance to monepantel, the latest anthelmintic in use, has occurred within less than four years of the product first being introduced, is certainly a disquieting warning sign (Raza et al., 2016a; Sales and Love, 2016).

Mechanisms of drug resistance can be divided into pharmacodynamic-mediated and pharmacokinetic-mediated. The first type includes processes such as a decrease in the amount of target macromolecules or changes in their structures, both of which cause a reduction in drug efficacy. In the case of benzimidazole anthelmintics, the occurrence of single nucleotide polymorphisms within three codons (167, 198, and 200) of the β-tubulin isotype-1 gene, which change the three-dimensional structure of the beta tubulin protein target of benzimidazoles, has been demonstrated as the primary mechanism of resistance (Lubega and Prichard, 1990; Lubega and Prichard, 1991; Chaudhry et al., 2015).

Pharmacokinetic-mediated mechanisms may involve decreased drug uptake, accelerated drug efflux and increased drug inactivation. In this way, the concentration of the active drug within parasite cells is decreased, a lower number of drug molecules are able to bind to target macromolecules, thus the drug effect is reduced (Fernando et al., 2016;

* Corresponding author. Dept. of Biochemical Sciences, Faculty of Pharmacy, Charles University, Heyrovského 1203, CZ-500 05 Hradec Králové, Czech Republic.
E-mail address: lenka.skalova@faf.cuni.cz (L. Skálová).

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Matouskova et al., 2016; Raza et al., 2016b). Pharmacokinetic-mediated drug resistance is based on an increase in the expression and activities of xenobiotic-metabolizing enzymes. In all organisms, these proteins serve as an efficient defense against the potential negative action of drugs and other xenobiotics. Several studies have described evidence of a direct association between xenobiotic-metabolizing enzymes and drug resistance in nematodes (reviewed in (Brophy et al., 2012; Matouskova et al., 2016)).

Our previous studies showed that *H. contortus* adults were able to metabolize the anthelmintic drugs albendazole (ABZ) and flubendazole (FLU) (Vokral et al., 2012; Vokral et al., 2013). ABZ-sulphoxide, ABZ-*N*-glucosides, FLU with reduced carbonyl group (FLU-R), FLU-*N*-glucosides and FLU-R-*O*-glucosides were identified in *H. contortus* adults incubated with ABZ and FLU, respectively. When the metabolisms of ABZ and FLU were compared in *H. contortus* strains sensitive and resistant to anthelmintics, a more pronounced glucosidation of both anthelmintics was found in the resistant strain. This finding indicates that drug deactivation via glucosidation could be one mechanism of resistance to benzimidazole anthelmintics in nematodes.

Nevertheless, a mix of adults of both sexes was used in these studies despite possible sex-differences in drug metabolism. Moreover, the analytical instruments available at the time of these experiments were less sensitive than present ones. Based on these particulars, the present study was designed to compare the metabolism of anthelmintics in females and males of *H. contortus* adults of an ISE (Inbred-Susceptible-Edinburg, MHco3) strain, which is susceptible to all main classes of anthelmintics (Roos et al., 2004), and a resistant IRE (Inbred-Resistant-Edinburgh; MHco5) strain (Yilmaz et al., 2017), which was directly developed from the ISE strain by imposing benzimidazole drug selection pressure. The metabolism of three benzimidazole anthelmintics was studied. In addition to ABZ and FLU, also ABZ-S-oxide (ABZSO; an active ABZ metabolite sold as ricobendazole, RCB) was included in this study. The use of high-sensitive UHPLC/MS/MS with a triple quadrupole mass analyzer allowed us to identify new metabolites of anthelmintics formed in *H. contortus* adults that have never been described previously.

2. Materials and methods

2.1. Chemicals and reagents

ABZ and RCB was purchased from Sigma-Aldrich (St. Louis, MO, USA). FLU was obtained from Janssen Pharmaceutica (New Brunswick, NJ, USA). Liquid sterile-filtered medium RPMI-1640 medium, HAM F12 medium, Williams' E medium, foetal calf serum and other chemicals (UHPLC, MS or analytical grade) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Collection of biological material

One susceptible ISE strain and one resistant IRE strain of *H. contorus* (obtained from Moredun Research Institute) were used in this study. The lambs were bred and used according to protocols which were evaluated and approved by the Ethics Committee of the Ministry of Education, Youth and Sports (Protocol MSMT-25908/2014–9). Six parasite-free lambs (3–4 months old) were orally infected with 5000 third stage larvae (L3) of *H. contortus*. Seven weeks after infection the animals were stunned and immediately exsanguinated in agreement with Czech slaughtering rules for farm animals. Adult nematodes were removed post mortem from the sheep abomasa using the agar method.

2.3. Experimental design

Freshly isolated living *H. contortus* adults were washed three times with phosphate-buffered saline (pH 7.4). Females and males were separated manually under a microscope. Males and females separately (ten nematodes per one sample) were placed in glass flasks with 5 mL of RPMI medium (pH 7.4, containing 60 μg mL^{-1} penicillin and 100 μg mL^{-1} streptomycin) and cultivated at 37 °C under humid atmosphere with 5% CO_2. At the beginning of the incubation, 2.5 mL of medium from each flask with the nematodes was removed and the same volume of fresh medium containing 10 μM FLU, ABZ or RCB (pre-dissolved in dimethyl sulfoxide (DMSO)) was added. The final concentration of DMSO in medium was 0.1% (*v/v*). After a 24-h incubation, the medium was placed into plastic tubes. The nematodes were washed three times with phosphate-buffer saline and were also transferred into the plastic tubes. The samples were frozen and stored at −80 °C. Chemical blanks (medium with anthelmintics, without nematodes) and biological blank samples (medium with nematodes, without anthelmintics) were prepared in the same way.

2.4. Sample preparation

The nematodes were homogenized repeatedly six-times for 10 s in cooled 0.1 M phosphate buffer (pH 7.4) using the FastPrep homogeniser, after which the homogenates were centrifuged at 3000 × g for 5 min. Supernatants of the homogenates as well as medium samples were extracted using the solid-phase extraction (SPE) as described previously (Stuchlikova et al., 2013). Dry samples were quantitatively reconstituted in a mixture of acetonitrile/water (30:70, *v/v*) using sonication and a vortex for 5 min. One microliter of reconstituted samples was injected into the UHPLC/MS system.

2.5. Proteins concentration measurement

The concentration of protein in the homogenates of the nematodes was measured using bicinchoninic acid assay according to Sigma-Aldrich protocols.

2.6. Analytical conditions of UHPLC-MS/MS

UHPLC (Nexera; Shimadzu, Japan) was optimized using a Zorbax RRHD Eclipse Plus 95 Å C18 column 150 × 2.1 mm, 1.8 μm (Agilent Technologies, Waldrbronn, Germany) at a temperature of 40 °C, flow rate 0.4 mL/min and injection volume 1 μl. The mobile phase consisted of water (A) and acetonitrile (B), both with the addition of 0.1% formic acid (MS grade). The linear gradient was as follows: 0 min–15% B, 8 min–40% B, 10 min–95% B followed by 1 min of isocratic elution. The QqQ mass spectrometer (LC-MS-8030 triple quadrupole mass analyzer; Shimadzu, Japan) was used with the following setting of tuning parameters: capillary voltage 4.5 kV, heat block temperature 400 °C, DL line temperature 250 °C, the flow rate and pressure of nitrogen were 12 l/min, respectively. ESI mass spectra were recorded in the range of *m/z* 50–1000 in the positive-ion mode, a greater sensitivity for the studied metabolites. The detected metabolites were identified based on the presence of the protonated molecules $[M+H]^+$ and the interpretation of their product ion spectra as described in Supplementary materials. The isolation width Δ*m/z* 2 and the collision energy 25 eV (found as optimal energy for fragmentation of studied metabolite ions) were used. Argon was the collision gas for MS/MS experiments. The standards of the potential metabolites were generally not commercially available, and they were not prepared due to the difficulties involved in their synthesis. For this reason, the amounts of metabolites were semi-quantified using a ratio of peak areas for the metabolites, with the area of the internal standard peak (mebendazole (MBZ)). In the homogenates of the nematodes, these ratios were normalized to milligram of total protein. All data are presented as arithmetic mean ± SD (n = 3).

2.7. Statistical analysis

The reported data are expressed as the mean ± S.D. (3–6 replicates). Statistical comparisons were carried out using the Student's t-