The metabolism of flubendazole and the activities of selected biotransformation enzymes in *Haemonchus contortus* strains susceptible and resistant to anthelmintics

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SUMMARY

Haemonchus contortus is one of the most pathogenic parasites of small ruminants (e.g. sheep and goat). The treatment of haemonchosis is complicated because of recurrent resistance of *H. contortus* to common anthelmintics. The aim of this study was to compare the metabolism of the anthelmintic drug flubendazole (FLU) and the activities of selected biotransformation enzymes towards model xenobiotics in 4 different strains of *H. contortus*: the ISE strain (susceptible to common anthelmintics), ISE-S (resistant to ivermectin), the BR strain (resistant to benzimidazole anthelmintics) and the WR strain (resistant to all common anthelmintics). *H. contortus* adults were collected from the abomasums from experimentally infected lambs. The *in vitro* as well as *ex vivo* experiments were performed and analysed using HPLC with spectrofluorimetric and mass-spectrometric detection. In all *H. contortus* strains, 4 different FLU metabolites were detected: FLU with a reduced carbonyl group (FLU-R), glucose conjugate of FLU-R and 2 glucose conjugates of FLU. In the resistant strains, the *ex vivo* formation of all FLU metabolites was significantly higher than in the susceptible ISE strain. The multi-resistant WR strain formed approximately 5 times more conjugates of FLU than the susceptible ISE strain. The *in vitro* data also showed significant differences in FLU metabolism, in the activities of UDP-glucosyltransferase and several carbonyl-reducing enzymes between the susceptible and resistant *H. contortus* strains. The altered activities of certain detoxifying enzymes might protect the parasites against the toxic effect of the drugs as well as contribute to drug-resistance in these parasites.

Key words: UDP-glucosyltransferase, carbonyl reductases, strain-differences, drug metabolism, helminths.

INTRODUCTION

In all organisms, drug biotransformation enzymes serve as an efficient defence against the potential negative action of xenobiotics, with the activities of biotransformation enzymes determining both desired and undesired effects of drugs. Although several types of biotransformation enzymes including oxidases, reductases, hydrolases, transferases and transporters have been described in various helminth species, the metabolism of anthelmintics in helminths has been relatively little investigated (Cvilink *et al*. 2009*a*).

Biotransformation of benzimidazole anthelminthics albendazole (ABZ) and triclabendazole (TCBZ) has been studied in several helminths, including *Haemonchus contortus*, with significant inter-species differences being observed (Solana *et al*. 2001; Mottier *et al*. 2004; Robinson *et al*. 2004; Alvarez *et al*. 2005; Cvilink *et al*. 2009*a*, *b*). In *H. contortus*, the conjugation of ABZ with glucose has been revealed (Cvilink *et al*. 2008*b*). Alvinerie and his coworkers (2001) have reported the formation of one metabolite of the anti-parasitic drug moxidectin in *H. contortus* homogenate incubations. On the other hand, *H. contortus* was not able to metabolize closantel, neither *in vitro* nor *ex vivo* (Rothwell and Sangster, 1997). When the biotransformation of flubendazole (FLU) was studied in *H. contortus*, the formation of FLU with a reduced carbonyl group (FLU-R) along with glucosides of FLU and FLU-R were found (Cvilink *et al*. 2008*a*, *b*).

The importance of investigating biotransformation enzymes in the helminth increased when several studies showed a direct association between biotransformation enzymes and drug resistance (Brennan *et al*. 2007). A significantly faster oxidation of TCBZ and a greater conversion of TCBZ-sulphoxide to TCBZ-sulphone have been found in resistant *Fasciola hepatica* as compared to susceptible individuals (Robinson *et al*. 2004; Alvarez *et al*. 2005). The metabolic inhibitors enhanced the drug susceptibility

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of a triclabendazole-resistant isolate of *F. hepatica* (Devine *et al*. 2010*a*, *b*).

Although drug-resistance in *H. contortus* is very common and several strains with different tolerance toward anthelmintics have been isolated, no information about differences in the biotransformation of anthelmintics among *H. contortus* strains has been made available. Moreover, while the results mentioned above clearly prove the importance of oxidation biotransformation enzymes in drug resistance, the contribution of other biotransformation enzymes to helminth resistance remains unknown. Therefore, the present study was designed to compare the biotransformation of FLU in *H. contortus* strains susceptible and resistant to anthelmintics. The benzimidazole drug FLU was chosen, as its biotransformation consists of the reduction of a carbonyl group and the formation of glucose conjugates in helminths (Cvilink *et al*. 2008*a*,*b*). Although FLU has not been commonly used in the treatment of haemonchosis, FLU efficacy against *H. contortus* has been described (Bártíková *et al*. 2010).

For this purpose 4 strains of *H. contortus* were used: the ISE strain (fully susceptible to anthelmintics), the ISE-S strain (resistant to ivermectin), the BR strain (resistant to benzimidazoles) and the WR strain (multi-resistant) *in vitro* as well as *ex vivo*. Assays of the *in vitro* activities of selected biotransformation enzymes have also been included in the study.

MATERIALS AND METHODS

Chemicals

FLU was purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). FLU-R was obtained from Janssen Pharmaceutica. Liquid sterile-filtered RPMI medium (Roswell Park Memorial Institute medium) and all other chemicals (HPLC or analytical grade) were obtained from Sigma-Aldrich (Prague, Czech Republic).

Collection of parasite material

One susceptible isolate of *Haemonchus contortus* –ISE and 3 resistant strains – White river (WR), ISE-S and benzimidazole-resistant strain (BR) have been used in this study. The *H. contortus* ISE strain is an anthelminthic-susceptible inbred type of the SE strain (Roos *et al*. 2004), which had been isolated from the field before benzimidazole anthelmintics were introduced to the market. BR strain is an inbred benzimidazole-resistant strain developed from the RE4 and SE population (Roos *et al*. 2004). The South African, multi-resistant WR isolate has been isolated from the field, and it has demonstrated resistance to ivermectin (30% efficacy at 0·2 mg/kg) as well as the benzimidazoles, rafoxanide and closantel (Van Wyk and Malan, 1988). The ISE-S isolate has been laboratory selected for ivermectin resistance in the Moredun Research Institute, Edinburgh, UK. Third-stage larvae (L3) of *H. contortus* strains were the kind gift of Dr Frank Jackson, Moredun Research Institute, Edinburgh, UK.

Twelve parasite-free lambs (3–4 months old) were orally infected with L3 larvae of *H. contortus*. Each animal obtained a suspension with 5000 L3 larvae. Seven weeks after infection the animals were stunned and immediately exsanguinated in agreement with Czech slaughtering rules for farm animals. Adult nematodes were removed from sheep abomasum using the agar method described by Van Wyk *et al*. (1980).

Preparation of subcellular fractions

The subcellular fractions from homogenates of *H. contortus* adults were prepared as described previously (Cvilink *et al*. 2008*a*). Protein concentrations were assayed using the bicinchoninic acid method according to the Sigma protocol.

Biotransformation of FLU ex vivo

Living nematodes were cultivated as described by Kotze and McClure (2001) with mild modification according to Cvilink *et al*. (2008*b*). At the beginning of incubation, 2·5 ml of medium was removed from each flask with nematodes and the same volume of fresh medium with anthelmintics was added. FLU was pre-dissolved in DMSO; the concentration of DMSO in medium was 0·1%. Nematodes were incubated in medium with anthelmintic (10 μM) for 24 h. In the first type of blank samples, medium with FLU but without nematodes was incubated. The second type contained drug-free medium with 0·1% DMSO and the parasite material. After incubation, medium was placed into the plastic tubes. The nematodes were repeatedly washed with phosphate-buffered saline and transferred into the plastic tubes. Samples were frozen and stored at −80 °C.

All samples were extracted by liquid/liquid (LL) extraction. Mebendazol (MBZ) at a concentration of 50 μM was used as an internal standard (IS). For LL extraction of medium, 10 μl of IS were added to 900 μl of medium and then the medium was divided into 3 equal parts. To each part, 30 μl of ammonium solution (concentrated, 25%, v/v) and 700 μl of ethyl acetate were added. Then samples were shaken (10 min, vortex) and centrifuged (1 min, 3000 ***g***). Supernatants from all 3 parts were put together and evaporated to dryness using an Eppendorf concentrator at 30 °C. Frozen nematodes were quickly homogenized using Sonopuls in 300 μl of redistilled water with 10 μl of IS. Then 30 μl of ammonium

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Table 1. Gradient programme for LC/MS analysis

Time (min)	A%	B%
0·00	90	10
10·00	40	60
11·00	20	80
12·90	20	80
13·00	90	10
25·00	90	10

Table 2. Basic information about detected analytes by LC-MS

Analyte name	SRM transition (m/z)	Retention time (min)	Collision energy for MS/MS (%)
FLU-R-gluc	478 → 298	9·16	50·0
FLU-gluc1	476 → 314	11·80	50·0
FLU-gluc2	476 → 314	12·15	50·0
FLU-R	316 → 284	10·27	50·0
MBZ (IS)	296 → 264	13·60	50·0

solution (concentrated, 25%, v/v) and 700 μl of ethyl acetate were added and samples were shaken (10 min, vortex) and centrifuged (1 min, 3000 ***g***). Supernatant was collected in the test tube and extraction was repeated. Supernatants from both extractions were combined and evaporated to dryness as described above. Dried extracts were stored (− 20 °C) until LC-MS analyses.

Liquid chromatography/mass spectrometry

For LC-MS measurements a slightly modified method as published by Cvilink *et al*. (2009*c*) was used. The liquid chromatography system consisted of a Surveyor MS pump and a Surveyor autosampler (both ThermoFinnigan, San Jose, CA, USA). A SymmetryShield RP 18 (2·1 × 100 mm, 3·5 μm; Waters, Milford, USA) column was used. The mobile phase consisted of solvent A (0·1% (v/v) aqueous formic acid) and solvent B (0·1% (v/v) formic acid in acetonitrile). The flow rate of the mobile phase was 120μl min^{-1}. The gradient used in this method is described in detail in Table 1.

The column compartment temperature was set to 40 °C. The MS/MS experiments were performed with an LCQ Advantage ion trap mass spectrometer (ThermoFinnigan, San Jose, CA, USA) equipped with an electrospray ionization (ESI) source. Before the experiments, the mass spectrometer was tuned for the best optimization using a direct infusion of FLU standard. All measurements were performed in positive ion mode. The analytes FLU-R, FLU-R-gluc, FLU-gluc1, FLU-gluc2, and MBZ were detected by monitoring the SRM transitions. For further information about analytes see Table 2.

Formation of FLU-R in vitro *and high performance liquid chromatography*

The reaction mixture (total volume of 0·3 ml) contained 50 μl of cytosol-like fractions containing 0·4–0·6 mg of proteins, FLU (10 μM) pre-dissolved in dimethyl sulfoxide (the concentration of DMSO in reaction mixture was 1%), NADPH (1 mM) and 0·1 M Na-phosphate buffer, pH 7·4. The blank samples contained 50 μl of 0·1 M sodium phosphate buffer, pH 7·4, instead of cytosols or 1% DMSO instead of FLU. In the inhibition study, the following inhibitors were tested: menadione (MEN), pyridinecarboxaldehyde (PCA), naloxone (NAL), metyrapone (MET). The concentration of inhibitors was 100 μM in the incubation mixture.

All incubations were carried out at 37 °C for 30 min under aerobic conditions. The product formation was linear up to 60 min. At the end of the incubation, 30μl of ammonium solution (concentrated) and 700 μl of cooled ethyl acetate were added, shaken (3 min, vortex) and centrifuged (10 min, 10 000 ***g***). Supernatants were evaporated and stored at −20 °C until HPLC analyses.

Chromatographic analyses were performed using an Agilent Technologies 1200 SL liquid chromatograph that consisted of a vacuum microdegasser, a 1200 SL binary pump, a 1200 SL plus autosampler, a TCC Infinity 1290 column thermostat and a 1200 SL diode-array detector. The chromatographic system was controlled by an Agilent ChemStation, version B.04.02 extended by a spectral module. The core-shell Ascentis® Express C18 (100 × 3·0 mm; 2·7 μm) column and the Supelguard Ascentis® C18 (20 × 4·0 mm; 3 μm) pre-column were utilized. An isocratic mobile phase was a mixture of KH_2PO_4 buffer (0·025 mol/l, pH 3) and acetonitrile (72 : 28) delivered at a flow rate of 0·8 ml/min. Detection conditions were found and described earlier (Kubíček *et al*. 2008). This analytical method was validated and a very low limit of FLU-R quantification (0·63 nmol/l) was found. Fifty μl of each sample was injected.

Formation of glucose conjugates in vitro

Microsome-like fractions from homogenates of *H. contortus* adults were pre-incubated with detergent slovasol (dissolved in redistilled water) for 20 min, at 4 °C. The amount of detergent was determined according to protein concentration to keep a ratio of 1:2 (detergent : protein). Pre-incubated fractions (200 μl containing 2·5–3·0 mg of proteins) were added to reaction mixtures (total volume 300 μl) containing 10 μM substrate FLU or FLU-R (pre-dissolved in DMSO), 100 μM UDP-glucose (dissolved in water) and 0·01M Tris/HCl buffer, pH 7·4. The blank samples contained 200 μl of 0·1 M sodium phosphate buffer, pH 7·4, instead of subcellular

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fractions. Incubations at 37 °C lasted 60 min. At the end of the incubation, 30 μl of ammonium solution (concentrated) and 700 μl of cooled ethyl acetate were added, shaken (3 min, vortex) and centrifuged (10 min, 10 000 ***g***). Supernatants were evaporated and stored at −20 °C until LC-MS analyses as described above.

Enzyme assays

Enzyme assays were performed in the subcellular fractions of the *H. contortus* homogenate. Each enzyme assay was performed in triplicate.

The activities of the reductases of the carbonyl group were tested using the following substrates: metyrapone, pyridinecarboxaldehyde, naloxone (all dissolved in redistilled water) and menadione (dissolved in ethanol). The amount of organic solvents in the final reaction mixtures did not exceed 1% (v/v). The concentrations of substrates, NADPH and potassium phosphate buffer, pH 6·8 were 1 mM, 0·3 mM and 0·1 M, respectively. The cytosolic fraction (50 μl, containing 0·26–0·32 mg of proteins) was added into the reaction mixture (total volume 1 ml). Spectrophotometric determination (detection wavelength 340 nm, 37 °C) of NADPH consumption in the reaction mixture served for the assessment of reductase activities (Ohara *et al*. 1995; Maté *et al*. 2008).

UDP-glucosyltransferase (UGlcT) activity was assayed in microsome-like fractions using p-nitrophenol as a substrate according to the method of Mizuma *et al*. (1982). The concentration of p-nitrophenol (pre-dissolved in redistilled water) was 3·3 μM. Absorbance was measured using the microplate reader Tecan Infinity M 200 (detection wavelength 415 nm).

Statistical analysis

The reported data are expressed as the mean ± S.D. of 3–6 replicates. Statistical comparisons were carried out using a non-parametric permutation (randomization) test (Microsoft Office Excel 2010). A probability of $P<0.05$ was considered statistically significant.

RESULTS

Biotransformation of FLU ex vivo

Living nematodes of 4 *H. contortus* strains were incubated in a medium with FLU. After 24 h, the medium and nematodes were collected separately and frozen. In the medium and homogenates of nematodes, FLU metabolites were identified and quantified using LC-MS. Four different FLU metabolites were detected: FLU with a reduced carbonyl group (FLU-R), a glucose conjugate of FLU-R and 2 glucose conjugates of FLU (Fig. 1).

FLU-R and FLU-R-glucoside (FLU-R-gluc) represented the major metabolites, while 2 FLU-glucosides (FLU-gluc1 and FLU-gluc2) were the minor ones. The amounts of metabolites were semi-quantified using a ratio of peak areas for the metabolites and the area of the internal standard peak, related to the wet mass of nematodes in the incubation. The formation of FLU-R metabolites during *ex vivo* incubations in the 4 *H. contortus* strains is compared in Fig. 2. The amount of FLU-R found in the medium and the homogenates was significantly higher in all the resistant strains as compared to the susceptible ISE strain.

The formation of FLU and FLU-R glucose conjugates is shown in Fig. 2. In homogenates, all 3 glucosides (FLU-R-gluc, FLU-gluc1 and FLU-gluc2) were semi-quantified; the amount of FLU-gluc2 in the medium was under the limit of quantification. In all resistant strains (both medium and homogenate), a significantly higher formation of glucose conjugates was observed in comparison to the susceptible ISE strain. The amount of FLU-R-gluc and both FLU-glucosides was increased, especially in the multi-resistant WR strain. This strain formed approximately 5 times more conjugates than the susceptible ISE strain.

Biotransformation of FLU in vitro

The ability of *H. contortus* to form FLU with a reduced carbonyl group (FLU-R) was tested in cytosol-like fractions prepared from homogenates of 4 *H. contortus* strains, as FLU reduction is mainly catalysed by cytosolic reductases (Cvilink *et al*. 2008*a*). The amounts of FLU-R were quantified using HPLC with spectrofluorimetric detection. *In vitro,* specific activities of FLU reductases were 0·12–0·16 pmol/min/mg of proteins. No significant differences in FLU reductase activities in individual strains were observed.

With the goal of revealing the potential competitive inhibitors and to estimate which enzymes participate in FLU reduction, the effect of selected model substrates of carbonyl-reducing enzymes on FLU reduction was tested. The obtained results (Fig. 3) showed menadione as an effective inhibitor and pyridinecarboxaldehyde as a mild inhibitor of FLU reduction in *H. contortus* cytosol-like fraction. Certain differences in the efficacy of both inhibitors on FLU reduction among strains were found. Metyrapone was shown to weakly decrease FLU-R formation only in the WR strain. No inhibitory effect of naloxone on FLU reduction was observed.

The *in vitro* formation of glucose conjugates of FLU and FLU-R was assayed in microsome-like fractions using LC-MS detection. FLU-R-gluc was

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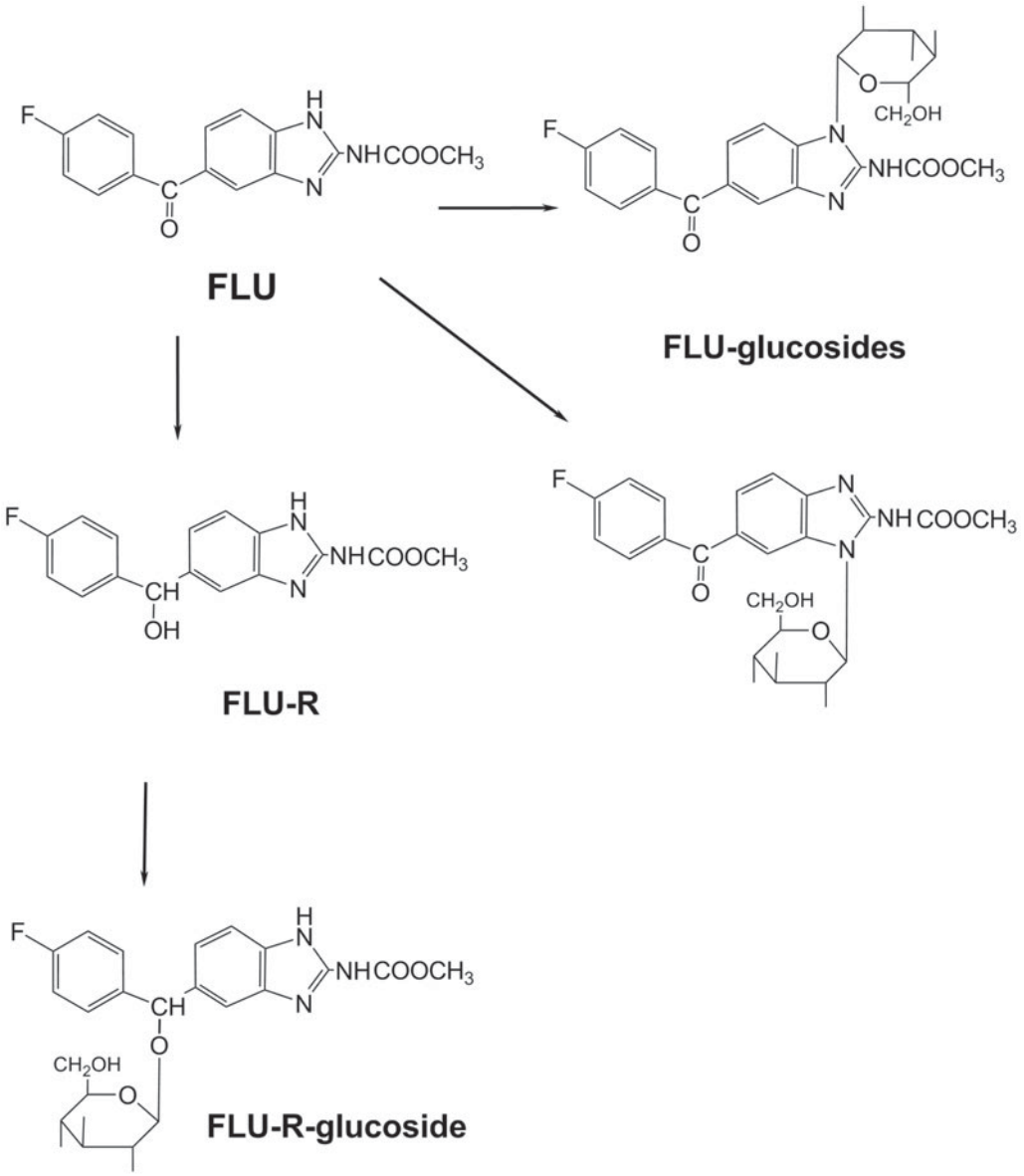

Fig. 1. Biotransformation pathway of FLU in *Haemonchus contortus*.

the only glucose conjugate formed *in vitro*. The comparison of FLU-R-gluc in the 4 strains is shown in Fig. 4. The results were expressed as areas under peaks because the standard of FLU-R-gluc was not available.

Enzyme assays in vitro

The specific activities of several carbonyl-reducing enzymes toward model substrates were assayed in cytosol-like fractions from nematode homogenates of the 4 *H. contortus* strains. The specific activities of UGlcT towards the model substrate *p*-nitrophenol were tested and compared in microsome-like fractions. The results are summarized in Table 3. Very high activities of pyridinecarboxaldehyde reductases, low activities of naloxone reductases and metyrapone reductases, and no activity of menadione reductases were detected. The specific activities of pyridinecarboxaldehyde reductases were significantly lower in the resistant BR and WR strains as compared to the ISE strain. The activities of metyrapone reductases were significantly lower in the ISE-S strain than in the ISE strain. On the other hand, the activities of naloxone reductases were found in the WR and ISE-S strains, while no activity was detected in the ISE and BR strains.

The formation of glucose conjugates with *p*-nitrophenol was not observed in the susceptible ISE strain, but all other strains showed a significant ability to form *p*-nitrofenol glucosides *in vitro*.

DISCUSSION

A direct association between the increased activity of oxidation biotransformation enzymes and drug resistance has been described only in *Fasciola hepatica* (Robinson *et al*. 2004; Alvarez *et al*. 2005).

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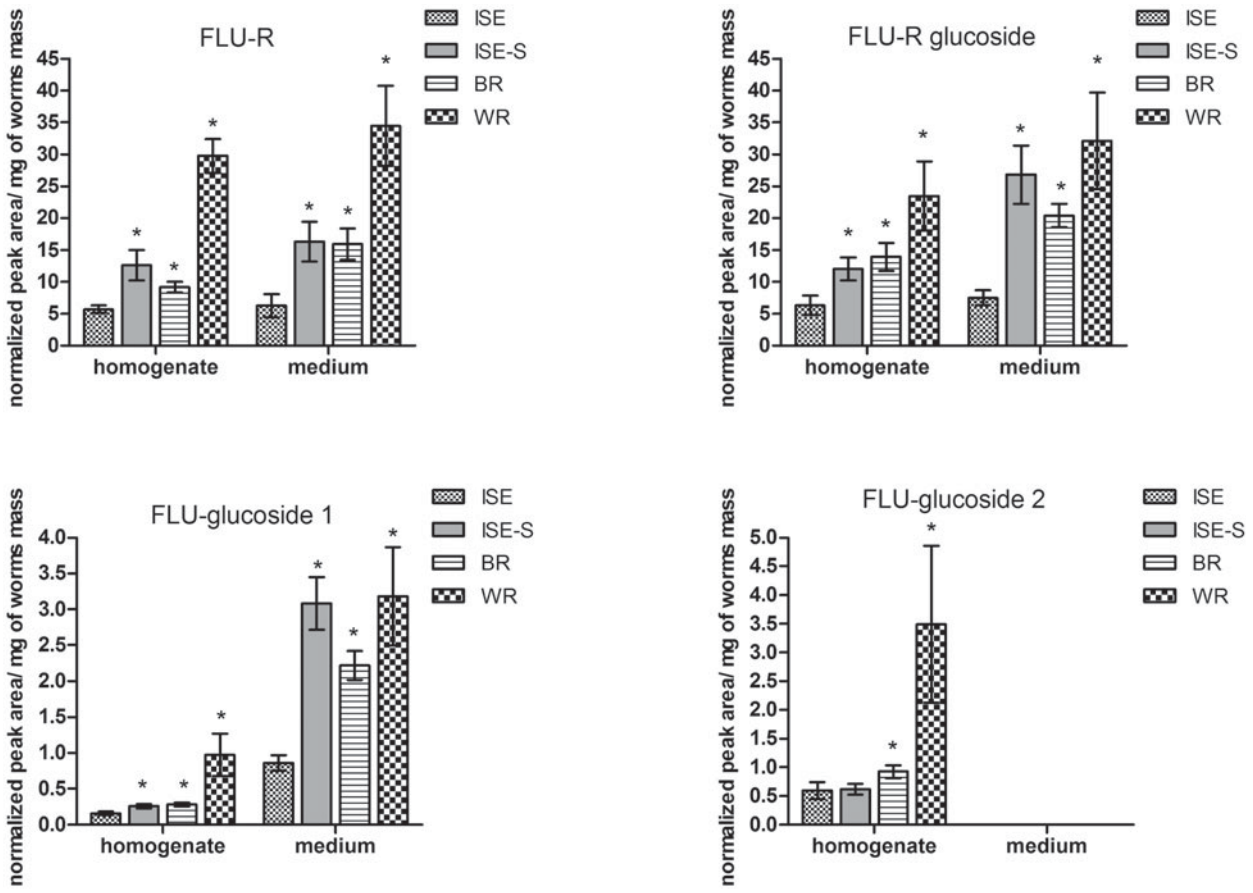

Fig. 2. Amounts of FLU-R and glucose conjugates of FLU-R (FLU-R-gluc) or FLU (FLU-gluc1 and FLU-gluc2) found in homogenates and medium after a 24-h *ex vivo* incubation of nematodes from 4 *Haemonchus contortus* strains with 10 μM FLU. FLU-gluc2 was detected only in the homogenate. See Materials and Methods section for further details.

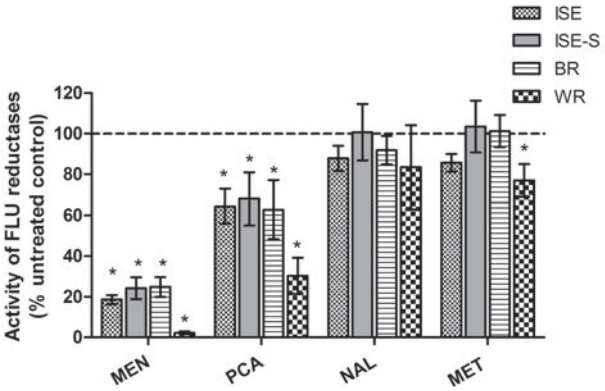

Fig. 3. Amounts of FLU-R analysed after *in vitro* incubation of cytosol-like fractions from 4 *Haemonchus contortus* strains with FLU (10 μM) and with the potential competitive inhibitors (selected model substrates of carbonyl-reducing enzymes) of FLU reductases (100 μM). Results are expressed as the percentage of the control (non-inhibited) reaction. The amount of FLU formed in non-inhibited reaction was 100%. MEN, menadione; PCA, pyridinecarboxaldehyde; NAL, naloxone; MET, metyrapone.

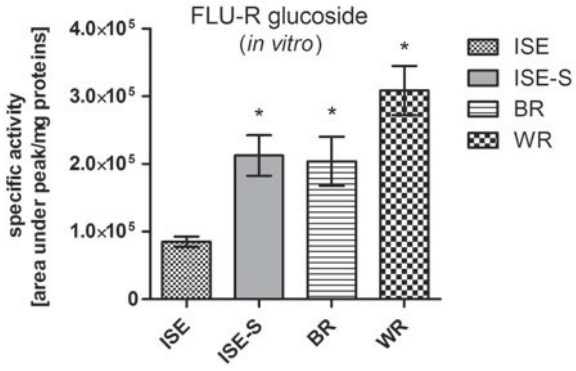

Fig. 4. Specific activities of UDP-glucosyltransferase (UGlcT) toward FLU-R assayed *in vitro* using microsome-like fractions from nematodes of 4 *Haemonchus contortus* strains. 10 μM FLU-R and 100 μM UDP-glucose were used. See Materials and Methods section for further details.

The aim of the present study was to find out whether the reduction and/or conjugation of anthelmintics differs between susceptible and resistant strains of *H. contortus* nematodes. The benzimidazole drug FLU was chosen, as its biotransformation consists of the reduction of a carbonyl group and the formation of glucose conjugates in *H. contortus* (Cvilink *et al.* 2008*b*). Four strains of *H. contortus* were used in the present study: the ISE strain (fully susceptible to anthelmintics), the ISE-S strain (resistant to

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Table 3. Specific activities of carbonyl-reducing enzymes toward selected substrates in cytosol-like fractions and of UDP-glucosyltransferase toward p-nitrophenol in microsomes-like fractions from *H. contortus* homogenates

(Data represent the means ± standard deviations.)

	Enzyme specific activities (nmol/min/mg of proteins)			
Substrate	ISE	WR	BR	ISE-S
4-pyridinecarboxaldehyde	144 ± 10	125 ± 10*	125 ± 7*	133 ± 4
naloxon	ND	1·5 ± 0·9*	ND	1·7 ± 1·3*
metyrapone	3·2 ± 0·2	2·1 ± 1·5	2·2 ± 1·5	2·0 ± 0·2
menadione	ND	ND	ND	ND
p-nitrophenol	ND	0·18 ± 0·06*	0·22 ± 0·11*	0·19 ± 0·10*

ND, Not detected.
* Statistically significant difference from sensitive ISE strain.

ivermectin), the BR strain (resistant to benzimidazoles) and the WR strain (multi-resistant).

Firstly, the biotransformation of FLU was tested and compared *ex vivo*. FLU metabolites were identified and semi-quantified using LC-MS. In all tested strains, 4 FLU metabolites were detected: FLU with a reduced carbonyl group (FLU-R), a glucoside of FLU-R (FLU-R-gluc) and 2 glucosides of FLU (FLU-gluc1 and FLU-gluc2). All means of biotransformation of FLU signify its deactivation, as FLU-R is less anthelmintically active than FLU (Bártíková *et al*. 2010) and conjugation with glucose or glucuronic acid always decreases the biological activity of xenobiotics. When amounts of FLU metabolites were compared among the 4 strains, significant differences were found. A lower formation of all metabolites in the susceptible ISE strain than in the resistant strains was observed. The most active in FLU biotransformation were the nematodes of the multi-resistant WR strain that formed approximately 4–6 times more FLU metabolites than the nematodes of the ISE strain. These results showed that the resistant strains possessed a higher ability to deactivate toxic xenobiotics than the susceptible strain.

Consequently, the *in vitro* assays were performed to specify the data obtained *ex vivo*. Cytosol-like fractions from the nematode homogenate were incubated with FLU and NADPH. When *in vitro* FLU-R formation was compared among the *H. contortus* strains, almost no inter-strain differences were found. Also the activities of carbonyl-reducing enzymes toward model substrates differed only mildly among *H. contortus* strains. The discrepancy between the *ex vivo* and *in vitro* results might be due to different conditions of the *in vitro* and *ex vivo* assays. In *in vitro* assays, very high (saturated) concentrations of substrate and coenzyme NADPH are used and almost all regulatory systems (compartmentation, transporters, modulation of enzymes expression etc.) are lacking. As *ex vivo* experiments to a greater degree mimic natural conditions, we consider the results obtained in these experiments to be more relevant to an actual *in vivo* situation. On the other hand, *in vitro* experiments remain indispensable and useful for many purposes, e.g. for enzyme identification and characterization.

With the goal of characterizing the enzyme(s) responsible for FLU reduction, typical substrates of individual carbonyl-reducing enzymes were used *in vitro* as competitive inhibitors of FLU reduction. 4-Pyridinecarboxaldehyde is a good substrate especially for human aldehyde reductase (AKR1A); naloxone is preferentially reduced by aldo/keto reductases (AKR1C); menadione is a typical substrate of human carbonyl reductase (CBR); metyrapon is reduced by AKR1C and CBR (Ohara *et al*. 1995; Maser and Opermann, 1997; Palackal *et al*. 2001; Gonzales-Covarrubias *et al*. 2008,). In *H. contortus*, FLU reduction was markedly inhibited by menadione and partly by 4-pyridinecarboxaldehyde. Using this result, the participation of CBR-like and AKR1A-like enzymes in FLU reduction in *H. contortus* could be estimated. Since the formation of FLU-R was also inhibited in the WR strain by metyrapone, this suggests the involvement of another enzyme (AKR1C-like enzyme) in FLU reduction in this strain. Certain inter-strain differences in the effect of inhibitors could be based on the different amount, affinity or activity of carbonyl-reducing enzymes in individual strains.

The second phase of FLU biotransformation in *H. contortus* consists of conjugation with glucose, which is catalysed by microsomal UGlcT. Therefore, the microsome-like fraction of nematodes and UDP-glucose was incubated with FLU or FLU-R, and the formed glucosides were analysed using LC-MS. *In vitro* only FLU-R-gluc was detected. Comparing the 4 strains, a significantly lower activity of UGlcT towards FLU-R was found in the microsomes of the susceptible ISE strain than in the resistant ones. When *p*-nitrophenol, a model substrate of UGlcT, was used no activity of UGlcT was detected in the ISE strain, while in the other strains this activity was evident. Taken together, the *in vitro* results

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confirmed the increased activity of UGlcT in the resistant *H. contortus* strains in comparison to the susceptible one. This finding, together with the higher formation of glucosides in resistant nematodes *ex vivo*, is the first indication that resistant strains of nematodes have an increased ability to deactivate anthelmintics via glucose conjugation.

CONCLUSIONS

In resistant strains of *H. contortus*, the *ex vivo* formation of all FLU metabolites was significantly higher than in the susceptible ISE strain. The *in vitro* data proved a significant increase in the activities of UGlcT in the resistant strains as opposed to the susceptible one. The increased activities of these detoxifying enzymes might protect the parasites against the toxic effect of the drugs, and thus contribute to drug resistance in these parasites.

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