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Effect of Flubendazole on Biotransformation Enzymes Activities in *Haemonchus contortus*

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Abstract: The aim of present project was to find out if *in vivo* contact of *Haemonchus contortus* with benzimidazole anthelmintic flubendazole (FLU) during treatment of its hosts (sheep) with low doses of FLU affects helminths' drug-metabolizing enzyme activities. Four groups of lambs, experimentally infected with *H. contortus*, were treated three-times orally with 0.0, 0.25, 0.50 or 1.00 mg per kg of body weight of FLU in three consecutive days. Twenty four hours after the last FLU dose, the nematodes were isolated, homogenized and subcellular fractions were prepared. In these subcellular fractions, biotransformation of FLU and the activities of carbonyl reducing enzymes and conjugation enzymes were assayed. The results showed that *H. contortus* enzymes were able to conjugate p-nitrophenol with glucose but not with glucuronic acid. The exposure of *H.contortus* to FLU (the highest FLU dose) caused a significant increase in activities of FLU reductases, D,L-glyceraldehyde reductases and glutathion S-transferases.

Keywords: Benzimidazoles, drug metabolism, enzyme modulation.

INTRODUCTION

Biotransformation enzymes in all organisms serve as an efficient defense against potential negative action of xenobiotics. Xenobiotics, including drugs, undergo biotransformation to achieve deactivation and easier excretion. Helminth biotransformation enzymes may thus diminish the final efficacy of anthelmintics. The ability to inactivate anthelmintics *via* biotransformation can to a certain extent protect these organisms against toxic effects of drugs [1-3].

A contact of organisms with toxic compounds may lead to induction of defensive enzymes, i.e. increased certain enzyme activities *via* increased expression or protein stabilization. Induction of several biotransformation enzymes by xenobiotics was observed in free living roundworm *Caenorhabditis elegans* [e.g. 4, 5]. Induction of catalase activities as a response to the oxidative stress was described in *H. contortus* [6]. In the case of anthelmintics, the induction of anthelmintics metabolizing enzymes could increase anthelmintics deactivation in parasites' bodies and by this way facilitate the surviving of some helminth' individuals exposed to anthelmintic therapy [7]. But till now, direct information about induction of helminth' biotransformation enzymes by contact of helminths with anthelmintics have not been reported yet.

The aim of present project was to find out if contact of *H. contortus* with benzimidazole anthelmintic flubendazole (FLU) during treatment of hosts (sheep) with FLU sub-optimal doses (non-lethal for all worms) affects biotransformation enzyme activities and FLU deactivation in survived helminths.

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MATERIALS AND METHODOLOGY

Chemicals

Flubendazole (FLU) and its two main metabolites (reduced flubendazole, hydrolyzed flubendazole) were provided by Janssen Pharmaceutica (Czech Republic). All other chemicals (HPLC or analytical grade) were obtained from Sigma-Aldrich (Czech Republic).

Experimental Infection of Sheep with *Haemonchus contortus*

H. contortus ISE strain used in this study was an anthelmintic-susceptible inbred strain of SE strain [8], which was isolated from the field before benzimidazole anthelmintics were on the market. The susceptibility of this *H. contortus* ISE strain to FLU was proved using larval development test [9]. Each of the sixteen parasite-free rams of sheep (*Ovis aries*), 5-6 months old, were orally infected with 5500 L_3 larvae of *H. contortus*. The development of *H. contortus* adults was coprologically monitored.

Treatment of Lambs with FLU

Flubenol (50% FLU premix, Janssen Pharmaceutica, Belgium) and Avicel RC-591 (microcrystalic cellulose; FMC, Belgium) were used for the preparation of aqueous suspension administered to animals [10].

Eight weeks after the infection, the sheep were divided into four groups (4 animals in each group). FLU-containing or FLU-free suspensions were administered orally using the

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loader for liquid pharmaceuticals. Each animal was treated with three doses administered every 24 hours in three consecutive days. The same volume of the suspension (0.75 mL) per kg of body weight was administered to all animals. The animals in Control group were treated with the same suspension without FLU. Each dose administered to animals in the remaining groups contained 0.25 mg (group FLU 0.25), 0.50 mg (group FLU 0.50) and 1.00 mg (group FLU 1.00) of FLU per kg of body weight per day. All experiments on animals were approved by the Ethical Committee of Faculty of Pharmacy, Charles University.

Collection of Nematodes

The nematodes were isolated from host twenty four hours after administration of the last FLU dose. The exposition of nematodes in abomasum to FLU lasted approx. 68-70hours. The animals were stunned and bleeding immediately in agreement with Czech slaughtering rules for farm animals. The abomasa were removed, ligated and transported in a warm bath (38°C) to the laboratory. Adult nematodes were isolated from the sheep abomasum using agar method [11]. Freshly isolated *H. contortus* adults were washed repeatedly in 0.1 M phosphate-buffered saline pH 7.4 and vivid (moving) worms were quickly frozen to -80°C.

Preparation of Subcellular Fractions from Nematodes

Nematodes (five grams from each sheep group) were thawed at a time and rapidly homogenized at the w/v ratio of 1:6 in 0.1 M sodium phosphate buffer, pH 7.4, using a Potter-Elvehjem homogeniser and sonication with Sonopuls (Bandelin, Germany). The subcellular fractions were isolated by fractional ultracentrifugation of the homogenate in the same buffer using the protocol for routine preparation of subcellular fractions from mammals' tissues [12]. Briefly, supernatant after two centrifugations (5000g for 20min. and 20000g for 60min.) was ultracentrifugated (105000g for 60 min.). Temperature was kept at 2-6°C during the whole procedure. Microsomes and cytosol like fractions were represented by 105 000 g sediment and supernatant, respectively, and were stored at -80°C. Protein concentrations were assayed using the bicinchoninic acid method according to the Sigma protocol.

Assay of FLU Biotransformation *In Vitro*

The cytosol like fractions were incubated in absence or in presence of FLU (0.5, 1.5, 3.0, 5.0 and 10.0 μM). Higher FLU concentration was not used as complete FLU solubility in reaction mixture could not be guaranteed. The reaction mixture (total volume of 0.3 mL) contained 100 μL of cytosol (containing 0.7-1.0 mg of proteins), FLU pre-dissolved in dimethyl sulfoxide (the DMSO concentration in the reaction mixture was 1%), NADPH (1 mM) and 0.1 M Na-phosphate buffer, pH 7.4. The blank samples contained 100 μL of 0.1 M sodium phosphate buffer, pH 7.4, instead of cytosol. The incubations were carried out at 37°C for 30 minutes under aerobic conditions. The product formation was linear up to 60 min. At the end of incubation, 30 μL of concentrated ammonium solution and 700 μl of cooled ethyl acetate were added, shaken (3 min., vortex) and centrifuged (10 min, 10000 g). Supernatants were evaporated and stored under -20°C until HPLC analyses.

HPLC Analysis of FLU and its Metabolites

Chromatographic analyses were performed with Shimadzu liquid chromatograph comprising a degasser GT-154, solvent delivery module LC-10ADvp, autoinjector SIL-10ADvp, column oven CTO-10Avp, UV/VIS photodiode array detector SPD-M10Avp, spectrofluorimetric detector RF-10AXL and system controller SCL-10Avp. The chromatograph was controlled by Shimadzu software CLASS-VP. A LiChroCART chromatographic column packed with LiChrospher 60 RP-select B (250 mm x 4 mm, 5 μm) and a precolumn (4 mm x 4 mm) packed with the same stationary phase were employed. Following chromatographic conditions were used: the mobile phase was a mixture of acetonitrile and 0.025 M KH_2PO_4 buffer, (3 : 7; v/v), pH = 3, delivered at a flow rate of 0.7 mL/min in isocratic mode. Using the photodiode array detector, the chromatograms were recorded at 246 nm and 300 nm (scan 195 – 380 nm). Using the spectrofluorimetric detector the excitation wavelength was 290 nm and emission wavelength was 320 nm. FLU-H, FLU-R and FLU were detected with the photodiode array detector. The spectrofluorimetric detector served for improving the sensitivity of FLU-R detection (FLU-H and FLU are not fluorescent). Fifty μL of each sample were injected onto the chromatographic column. The duration of the complete analysis was 22 minutes. This analytical method was validated [13] and very low limit of FLU-R quantification (0.63 $nmol.L^{-1}$) was found. Prior to the *in vitro* incubations, the cytosolic fractions of *H. contortus* from FLU treated animals were tested for the presence of FLU and its metabolites. No traces of FLU, FLU-R or other FLU metabolites were found in either cytosolic fraction.

Enzyme Assays

Enzyme activities were assayed in the cytosol or microsomes like fractions of *H. contortus* homogenate. Each enzyme assay was performed in triplicates. The amount of organic solvents in the final reaction mixtures did not exceed 0.1% (v/v).

The activities of carbonyl reductases/dehydrogenases were tested using the following substrates: 4-pyridinecarboxaldehyde (dissolved in redistilled water), D,L-glyceraldehyde (dissolved in DMSO), and 1-acenaphthenol (dissolved in DMSO). The concentrations of substrates, NADPH (or $NADP^+$ for 1-acenaphthenol) and potassium phosphate buffer, pH 6.0 (or TRIS/HCl buffer, pH 8.0 for 1-acenaphthenol), were 1 mM, 0.3 mM and 0.1 M, respectively. The 10-50 μL of cytosolic fractions (containing 6.4-7.8 mg of proteins per mL) were added into the reaction mixture (total volume 1 mL). Spectrophotometric determination (detection wavelength 340 nm, 25°C) of NADPH consumption or formation in the reaction mixture served for the assessment of reductase/dehydrogenase activities [14-16].

The microsomal UDP-glucuronosyl transferase activity was assayed following the method by Mizuma *et al.* [17]. Microsomes were preincubated with the Slovasol detergent at 4°C for 20 minutes. The reaction mixture (total volume of 0.1 mL) contained 10 μL of microsomes (0.12-0.14 mg of protein), 0.33 mM UDP-glucuronic acid and 166.8 μM *p*-nitrophenol (dissolved in redistilled water) in 0.1 M Tris/HCl buffer (pH 7.4). After 20 minutes of incubation at 37°C, the

reaction was stopped by addition of 50 µL 3% trichloroacetic acid (v/v). After shaking and centrifugation (3 min., 2000 g), 50 µL of the supernatant were mixed with 50 µL 1M NaOH. The absorbance was measured using a BioRad microplate reader (detection wavelength 415 nm). The same procedure was used for the testing of microsomal UDP-glucosyl transferase activity using UDP-glucose instead of UDP-glucuronic acid.

The cytosolic glutathione S-transferase (GST) activities were assayed using 1-chloro-2,4-dinitrobenzene as a substrate (dissolved in ethanol). The reaction mixture (total volume 1 mL) contained 10 µL of cytosol (0.14-0.18 mg of protein), 1 mM reduced glutathione, 1 mM 1-chloro-2,4-dinitrobenzene in 0.1 M Na-phosphate buffer (pH = 6.5). The reaction mixture was shaken and the absorbance was measured spectrophotometrically at 340 nm four times in 60 second intervals [18].

RESULTS AND DISCUSSION

Benzimidazole anthelmintic FLU is widely used for antiparasitic control in pigs, chicken, turkeys, game birds and domestic carnivores. Efficacy of FLU against roundworms has been also proved in the parasitoses treatment in small ruminants [19] and it is intended for therapy of haemonchosis in sheep.

Metabolism of FLU in *H. contortus* subcellular fractions was firstly studied in our previous project [20]. We found that *H. contortus* enzymes metabolized FLU *via* reduction of its carbonyl group (Fig. **1**) The ability of *H. contortus* enzymes to reduce carbonyl group of xenobiotics was further confirmed using model substrates (D,L-glyceraldehyde, metyrapone, daunorubicin).

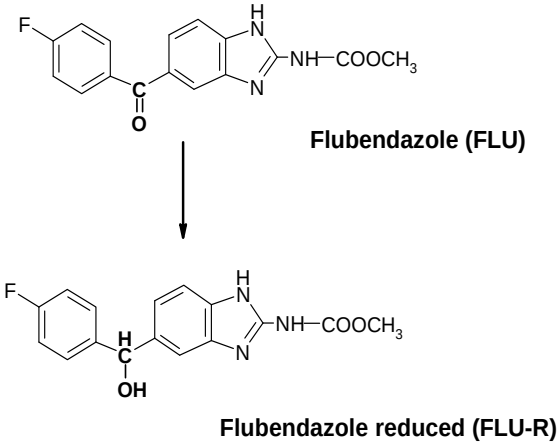

Fig. (1). Structure of flubendazole (FLU) and its metabolite reduced FLU (FLU-R).

In farm animals, modulation of biotransformation enzymes *via* FLU treatment was found [12] but no information about FLU effect on helminth´ biotransformation enzymes has been available. The present project was designed to evaluate if repeated *in vivo* contact of *H. contortus* with low doses of FLU affects *H. contortus* biotransformation enzymes and FLU metabolism *in vitro*. Nematodes were obtained from control sheep (Control) or sheep treated in three previous days with 0.25 (group FLU 0.25), 0.50 (group FLU 0.50) and 1.00 (group FLU 1.00) mg of FLU per kg of body weight per day. The highest dose used lies in range of one third and one tenth of doses, which are recommended as therapeutic ones in monogastric animals [21]. Repeated treatment of animals in three consecutive days was chosen to achieve the exposition time enough for induction of biotransformation enzymes.

Generally, xenobiotic modulates (induces or inhibits) mainly those biotransformation enzymes that catalyze its biotransformation. In presented study, the main attention was paid on carbonyl reductases modulation as FLU is metabolized *via* carbonyl reduction in *H. contortus. In vitro* activities of carbonyl reductases toward selected model substrates [15, 16, 22, 23] were tested in *H. contortus* cytosol like fractions. The results proved the ability of *H. contortus* enzymes to reduce the carbonyl group of organic compounds. The contact of nematodes in hosts with the highest FLU dose (1.00 mg per kg per day) led to a significant increase only in DL-glyceraldehyde reductase (GALR) activity (see Table **1**).

With aim to know if previous contact of helminths with FLU in hosts affect FLU reduction in helminths, the activities of FLU reductases were tested in cytosol like fractions from each nematodes´ groups. The results were presented in Fig. (**2**). The FLU reductases activities were significantly increased (by 25-50%) in FLU 1.00 group comparing to the Control group when lower concentrations of substrate (FLU) were used in *in vitro* incubations. But this increase was not detected when a higher concentration of substrate was used in incubations. As several enzymes are able to reduce carbonyl containing xenobiotics [24] this discrepancy could be explained by the participation of at least two enzymes in FLU reduction. Contact of living helminth with FLU probably caused the induction of the one FLU reductase that has a higher affinity but lower capacity to FLU. The activity of second FLU reductase (with lower affinity and higher capacity) was probably not affected and due this fact the induction effect of FLU came out only at lower FLU concentrations.

When FLU biotransformation was studied in *H. contortus ex vivo*, two phase II metabolites of FLU - conjugates of FLU and FLU-R with glucose - were discovered [25]. With respect to this fact, the conjugation enzyme assays were also included in the present project.

H. contortus ability to conjugate the model xenobiotic substrate (p-nitrophenol) with UDP-glucose or UDP-glucuronic acid was tested in microsomal fractions. The results proved that *H. contortus* enzymes use glucose but not glucuronic acid for conjugation of xenobiotics. This finding is interesting as glucose conjugation, common metabolic pathway in plants, occurs rarely in animal kingdom. Focusing on helminths, only O'Hanlon and coworkers [26, 27] reported glucose conjugation in ecdysteroids metabolism in nematodes *Ascaris suum* and *Parascaris equorum*. The contact of *H. contortus* with FLU did not affect UDP-glucosyl transferase activity towards p-nitrophenol since any significant differences among control group and all FLU groups were not found.

Table 1. Specific Activities of D,L-Glyceraldehyde Reductase (GALR), Acenaphthenol Dehydrogenase (ACND) Nad Glutathione S-Tranferases in *H. contortus* from Sheep Without FLU (Control) and FLU Treated Sheep (FLU 0.25, FLU 0.50 and FLU 1.00)

Specific activity \(nmol/min/mg of proteins)	Control	FLU 0.25	FLU 0.50	FLU 1.00
GALR	9.1 ± 1.0	8.2 ± 3.0	11.3 ± 2.4	12.2 ± 0.7*
ACND	4.1 ± 0.4	4.8 ± 0.2	5.5 ± 0.3*	3.4 ± 0.3*
GST	257 ± 17	260 ± 10	322 ± 67	340 ± 73*

*Statistically significant difference to Control group ($p \leq 0.05$).

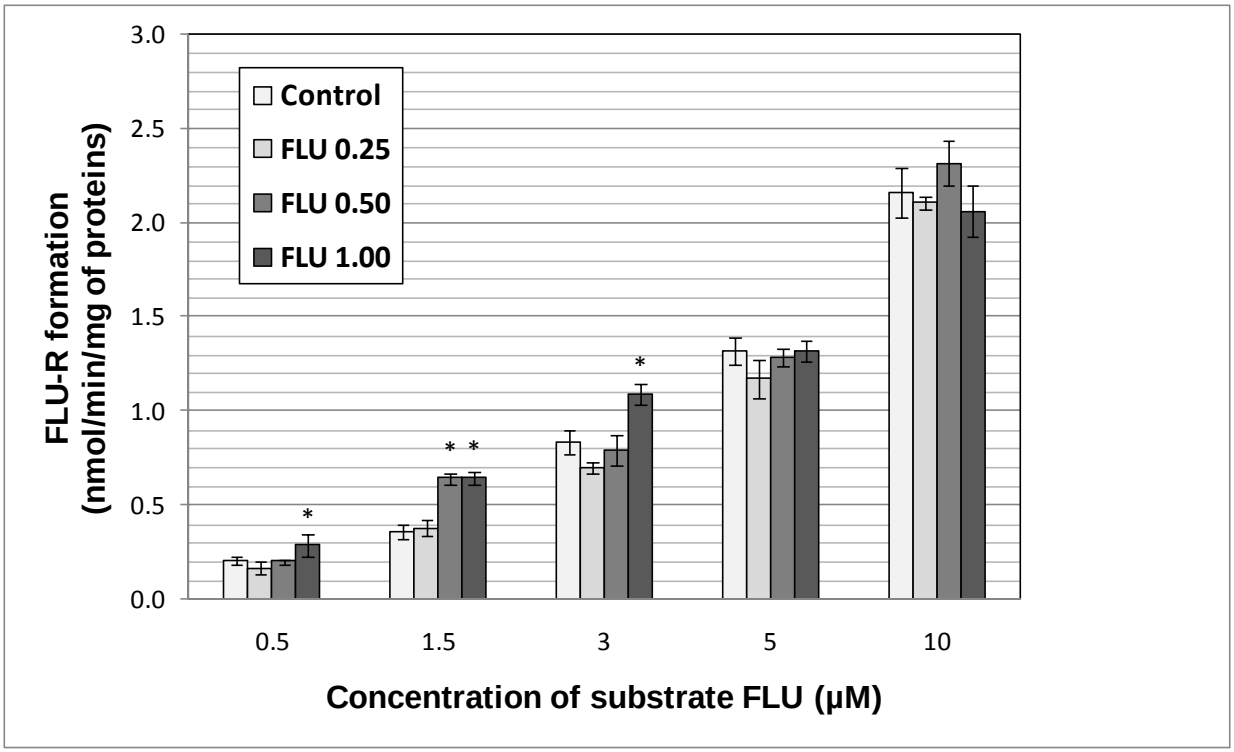

Fig. (2). Specific activities of *Haemonchus contortus* cytosolic reductases towards flubendazole at various concentrations.

Data represent the means ± S.D. (n=4).

*Statistically significant difference to Control group ($p \leq 0.05$).

While nothing is known about helminth UDP-glucosyl transferases, other conjugation enzymes, glutathione S-transferases (GSTs), have been studied intensively and present knowledge of structure and localization of helminth GSTs is large [e.g. 28]. In spite of this fact, information about GSTs mediated xenobiotics conjugation is rare. Lo and colleagues [29] showed that extracts from *Schistosoma mansoni* efficiently conjugated synthetically prepared electrophilic compounds with glutathione. Vande Waa *et al.* [30] found GST activity towards model substrate (1-chloro-2,4-dinitrobenzene, CDNB) in *Schistosoma mansoni*. In present project, relatively high activity in CDNB conjugation with glutathione was observed in *H. contortus* cytosolic fractions. This specific activity was similar to those found in hepatic cytosol from several mammalian species [31].

When *H. contortus* nematodes were in contact with the highest FLU concentration *in vivo* (group FLU 1.00), helminth' *in vitro* GST activity was significantly increased, approximately by 30% (see Table **1**) The induction of GST activity could be caused by oxidative stress arising during FLU effect over the parasite. Classical inducer, phenobarbital, induced GST activity in *Hymenolepis diminuta*, *Echinococcus granulosus* and *Schistosoma mansoni* and other model inducers, 3-methylcholanthrene and butylated hydroxyanisole, significantly induced GST activity against CDNB in *Schistosoma mansoni* [30, 32, 33]. The increase in GST activities caused by the aforementioned inducers was much more substantial than that caused by FLU in the presented study. On the other hand, FLU is the only anthelmintic, the ability of which to induce the GST was found in helminths.

Although the observed increase of *in vitro* carbonyl reducing enzymes and GST activities in nematodes previously *in vivo* exposed to FLU was only slight (but significant), this is the first indication that a contact of *H. contortus* with FLU in hosts may lead to an induction of some biotransformation

enzyme activities of parasites. The increased ability to deactivate FLU may facilitate the surviving of some *H. contortus* worms under anthelmintic therapy. If more worms survive, the reproduction of those specimens with higher FLU tolerance is more probable. By this way, induction of biotransformation enzymes might represent additional mechanism contributing to development of drug-resistant *H. contortus* strain.

ACKNOWLEDGEMENTS

Financial support of this project was provided by Czech Science Foundation, Grant No. P502/10/0217.

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Received: March 18, 2010 Revised: June 16, 2010 Accepted: June 16, 2010