The activity of drug-metabolizing enzymes and the biotransformation of selected anthelmintics in the model tapeworm *Hymenolepis diminuta*

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SUMMARY

The drug-metabolizing enzymes of some helminths can deactivate anthelmintics and therefore partially protect helminths against these drugs' toxic effect. The aim of our study was to assess the activity of the main drug-metabolizing enzymes and evaluate the metabolism of selected anthelmintics (albendazole, flubendazole, mebendazole) in the rat tapeworm *Hymenolepis diminuta*, a species often used as a model tapeworm. *In vitro* and *ex vivo* experiments were performed. Metabolites of the anthelmintics were detected and identified by HPLC with spectrofluorometric or mass–spectrometric detection. The enzymes of *H. diminuta* are able to reduce the carbonyl group of flubendazole, mebendazole and several other xenobiotics. Although the activity of a number of oxidation enzymes was determined, no oxidative metabolites of albendazole were detected. Regarding conjugation enzymes, a high activity of glutathione S-transferase was observed. A methyl derivative of reduced flubendazole was the only conjugation metabolite identified in *ex vivo* incubations of *H. diminuta* with anthelmintics. The results revealed that *H. diminuta* metabolized flubendazole and mebendazole, but not albendazole. The biotransformation pathways found in *H. diminuta* differ from those described in *Moniezia expanza* and suggest the interspecies differences in drug metabolism not only among classes of helminths, but even among tapeworms.

Key words: biotransformation enzymes, drug metabolism, albendazole, flubendazole, mebendazole, liquid chromatography/mass spectrometry.

INTRODUCTION

Tapeworms cause serious infections that afflict humans as well as a lot of animal species and cause health and economic losses (Webbe, 1994; Matossian *et al*. 1977; White, 2000; Budke *et al*. 2006; Moro *et al*. 2011). Therefore, research into an effective defence against these parasites remains a central priority in the scientific community.

Hymenolepis diminuta (*H. diminuta*), also known as the rat tapeworm, is a common cestode parasite in rats, mice and other rodents. Although *H. diminuta* is spread worldwide, it rarely occurs in humans (Wiwanitkit, 2004). In spite of the fact that *H. diminuta* is of little direct medical importance, it is of great scientific interest as a laboratory model in the study of physiology, biochemistry and immunology of tapeworms and for evaluation of the effects of anthelmintic drugs (Muller and Wakelin, 2002; Horak and Klimes, 2007; Hitchen *et al*. 2009; Cunningham and Olson, 2010).

Several anthelmintics are used in the control of tapeworm infections. Praziquantel and niclosamide represent drugs of choice for this helminthosis (Cohen and Mackey, 1977; Jones, 1979; Tanowitz *et al*. 1993; Marangi *et al*. 2003). The benzimidazole drugs (BZD), albendazole (ABZ), mebendazole (MBZ), flubendazole (FLU), thiabendazole and cambendazole are also effective in lessening tapeworm burden (McCracken and Taylor, 1983; Maki and Yanagisawa, 1985; McCracken *et al*. 1992; Ostlind *et al*. 2004).

Unfortunately, the efficacy of anthelmintics is not sufficient in some cases of cestode infection (Slocombe, 1979; Hussain *et al*. 2004). Tapeworms, as well as other helminths, may possess mechanisms which protect them against the negative effects of anthelmintics and other xenobiotics. This defence system is based on the activities of drug-metabolizing enzymes (i.e. biotransformation enzymes and drug-transporters) that are able to metabolize (inactivate) anthelmintics and/or accelerate drug efflux from the helminth's body. In this way the helminth's drug-metabolizing enzymes could diminish the toxic effect of the drugs and other xenobiotics, thus decreasing the effectiveness of the control and treatment of helminth infections (Robinson *et al*. 2004; Kotze *et al*. 2006).

The drug-metabolizing enzymes of helminths have not been investigated to a great extent so far (Cvilink

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et al. 2009*b*). Reductases, hydrolases and conjugation enzymes have been considered to be important biotransformation enzymes in helminths (Barrett, 1997; Rossjohn *et al*. 1997), but several examples of the oxidative metabolism of xenobiotics have been also found in certain helminth species (Cvilink *et al*. 2009*b*). In *H. diminuta*, only sporadic information about drug-metabolizing enzymes (Munir and Barrett, 1985), and no information on the biotransformation of anthelmintics has been reported. Therefore, the present study was designed to evaluate the metabolism of several anthelmintics and to determine the activities of the main biotransformation enzymes in *H. diminuta in vitro* and *ex vivo*. The results obtained were then compared to accessible data for other cestodes with the goal of evaluating interspecies differences in drug metabolism of cestodes.

MATERIALS AND METHODS

Chemicals

Albendazole was purchased from Sigma-Aldrich (Prague, Czech Republic). Albendazole sulphoxide (ABZ.SO) and albendazole sulphone (ABZ.SO_2) were obtained from Toronto Research Chemicals Inc. (North York, ON, Canada). Flubendazole (FLU), reduced flubendazole (FLU-R), mebendazole (MBZ), and reduced mebendazole (MBZ-R) were provided by Janssen Pharmaceutica (Prague, Czech Republic). Liquid sterile-filtered RPMI 1640 medium (Roswell Park Memorial Institute medium) and all other chemicals (high performance liquid chromatography (HPLC) or analytical grade) were obtained from Sigma-Aldrich. SOD Assay Kit-WST (Dojindo, Tabaru, Japan) was purchased from Probior (München, Germany).

Biological material

H. diminuta adults were obtained from young male rats (*Rattus norvegicus*), previously infected with 6–8 cysticercoids. The pre-patent period for *H. diminuta* in our laboratory was about 16 days. As an intermediate host, beetles *Tenebrio molitor* were used. Larval and adult stages of beetles were kept together and fed by flour and granulated feed. Water was served 3 times a week. For the infection of intermediate hosts, rat feces or last segments (1 cm) of adult tapeworm were used. After 12 days, beetles were dissected and obtained cysticercoids were transferred to the rats by stomach probe. Two months post-infection, rats were decapitated and adult tapeworms were flushed out from small intestine by phosphate buffer saline (0·01 M, pH 7·4). The obtained tapeworms were washed 3 times with the same buffer and were either kept in a freezer (−80 °C) until the subcellular fractions were prepared or used immediately in *ex vivo* experiments.

Preparation of subcellular fractions

Microsomes-like, cytosol-like and mitochondria-like fractions were obtained from tapeworm homogenates using fractional ultracentrifugation. For more details, the reader is referred to Bartikova *et al*. (2010*b*). Protein concentrations in subcellular fractions were assayed using the bicinchoninic acid method according to the protocol of Smith *et al*. (1985).

Enzyme assays

Enzyme activities were assayed in the cytosol-like, mitochondria-like and microsomes-like fractions of *H. diminuta* homogenate. Enzyme assays were performed in 3–6 parallel measurements for each sample. The amount of organic solvents in the final reaction mixtures did not exceed 1% (v/v).

The methods for determination of peroxidase (Px) and catalase (CAT) activities have been described in detail by Bartikova *et al*. (2010*b*). The assay of superoxide dismutase (SOD) was performed using SOD Assay Kit-WST according to the general protocol. The inhibition activity of SOD on $O_2^{\cdot -}$ formation leads to lower formazan production, which can be determined by a colourimetric method. The activity of SOD is expressed as the decrease ($nmol \cdot min^{-1} \cdot mg^{-1}$) of formazan production.

The activities of carbonyl-reducing enzymes were tested in cytosol-like fraction using the following substrates dissolved in redistilled water: 1 mM metyrapone, 1 mM 4-pyridinecarboxaldehyde and 0·01 mM daunorubicin (at pH 6·0 and pH 8·5). Spectrophotometric determination (detection wavelength 340 nm, 25 °C) of NADPH consumption in the reaction mixture served for the assessment of reductase activities (Maser, 1995; Ohara *et al*. 1995; Palackal *et al*. 2001). Enzymatic activities for the model substrate acenaphthenol and menadione were assayed spectrofluorimetrically using Perkin-Elmer LS 50 B luminescence spectrophotometer. For the acenaphthenol dehydrogenases, the excitation wavelength 340 nm and emission wavelength 480 nm were used. The velocity of substrate dehydrogenation was measured as an increase in fluorescence caused by the reduction of co-factor ($NADP^+$). A final 1·0 ml reaction contained 1·5 mM acenaphthenol dissolved in DMSO, 1·0 mM $NADP^+$, 10 μl of subcellular fraction and 0·1 M Tris-HCl buffer, pH 8·9 (Palackal *et al*. 2001). The assay with substrate menadione was carried out at 380 nm (excitation) and 460 nm (emission). Biotransformation of menadione is coupled with NADPH oxidation which is recorded by the decrease of fluorescence. The 1 ml reaction mixture consisted of 0·5 mM menadione (in ethanol), 0·25 mM NADPH, 50 μl of subcellular fraction and 0·1 M K-phosphate buffer, pH 7·4 (Maté *et al*. 2008). Reductases of oracin were assayed as described previously (Szotakova *et al*. 2000; Wsol *et al*. 2007).

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The UDP-glucuronosyl transferase (UGT) and UDP-glucosyl transferase (UGlcT) activity in microsome-like and mitochondria-like fractions was assayed according to the method of Mizuma *et al*. (1982). The final concentration of *p*-nitrophenol (in redistilled water) was 3·3 μM. Absorbance was measured using a microplate reader Tecan Infinity M 200 (detection wavelength 415 nm).

Cytosolic glutathione S-transferase (GST) activity was determined using 1 mM 1-chloro-2,4-dinitrobenzene as a substrate (in ethanol). The increase of absorbance at 340 nm was measured spectrophotometrically (Habig and Jakoby, 1981).

In vitro *biotransformation of ABZ, ABZ.SO, FLU and MBZ and drug extraction*

The subcellular fractions were incubated with ABZ, ABZ.SO, FLU or MBZ and coenzyme NADPH. The total volume of the reaction mixture (0·3 ml) contained 100 μl of fractions (0·3–0·7 mg of proteins), anthelmintics (1·0 and 10·0 μM) pre-dissolved in DMSO (FLU, MBZ) or methanol (ABZ, ABZ.SO), 1·0 mM NADPH and 0·1 M sodium phosphate buffer (pH 7·4). The concentrations of anthelmintics to be used were established on the basis of previous experiments (1 μM concentration was the lowest concentration which permitted detection of the metabolites, while 10 μM represents the highest concentration in which tapeworms were able to survive in *ex vivo* experiments). The first type of blank samples contained 100 μl of 0·1 M sodium phosphate buffer (pH 7·4) instead of fractions, in the second one, the fractions were incubated with DMSO only (or methanol) without drugs. The concentration of solvents in all reaction mixtures and blank samples was 1% (v/v). In the kinetic study of FLU and MBZ reduction, the concentrations range 1·0 to 15·0 μM of FLU or 1·0 to 20·0 μM of MBZ, 1 mM NADPH and 100 μl of cytosol were used. The incubations were carried out at 37 °C for 30 min under aerobic conditions. At the end of the incubation, 30 μl of ammonium solution (concentrated, 25% v/v) and 700 μl of ethyl acetate were added. After shaking (3 min, vortex) the mixture and centrifugation (3 min, 5000 ***g***), the supernatants were removed and subsequently evaporated. Samples were stored below −20 °C until HPLC or liquid chromatography/mass spectrometry (LC/MS) analyses.

Ex vivo *cultivation of* H. diminuta *with anthelmintics and drug extraction*

Tapeworms, having been washed 3 times in phosphate buffered saline with penicillin (60 $\mu g \cdot ml^{-1}$) and streptomycin (100 $\mu g \cdot ml^{-1}$), were cultivated individually in plastic flasks with 10 ml of RPMI 1640 medium (pH 7·4, with 60 $\mu g \cdot ml^{-1}$ penicillin and 100 $\mu g \cdot ml^{-1}$ streptomycin) containing one of the drugs (ABZ, ABZ.SO, FLU or MBZ; 1 μM and 10 μM) at 38 °C in a humid aerobic atmosphere with 5% CO_2. FLU and MBZ were pre-dissolved in DMSO, ABZ and ABZ.SO in methanol. Final concentration of DMSO (or methanol) in RPMI medium was only 0·1% (v/v) to prevent any harmful effects on living tapeworms. Two types of blank samples, medium with anthelmintics but without parasites, and control tapeworms cultivated in drug-free RPMI medium with DMSO (or methanol), were prepared. Tapeworms with drugs and blank samples were incubated for 24 h. The viability of tapeworms was assessed as visible movement of parasites. After incubation, medium (900 μl) was placed into the microtubes and the tapeworms were rinsed thoroughly 3 times with phosphate-buffered saline and then transferred to the tubes. The samples were frozen and stored at −80 °C until processed for HPLC or LC/MS.

Prior to HPLC analysis, tapeworms were quickly homogenized (up to 20 s) in 0·1 M phosphate buffer (pH 7·4) using Sonopuls (Bandelin, Germany). The medium or homogenate (0·3 ml) was brought to alkaline pH with 30 μl of ammonium solution (concentrated, 25% v/v). After the addition of 700 μl of ethyl acetate, the mixture was shaken (3 min, vortex) and centrifuged (3 min, 5000 ***g***). Supernatants were evaporated and dry samples stored below −20 °C until HPLC analysis.

The description of sample preparation for LC/MS is given below (SPE and sample preparation).

HPLC analysis

The HPLC analysis was carried out using a Shimadzu chromatograph. Assay of ABZ and its metabolites was performed according to Cvilink *et al*. (2009*b*). Under these chromatographic conditions, the retention times for ABZ and its metabolites were 5·1 min (ABZ.SO), 7·1 min ($ABZ.SO_2$), and 17·2 min (ABZ). The limit of quantification for ABZ, ABZ.SO, and $ABZ.SO_2$ was 120·0, 2·0, and 0·07 pmol/ml, respectively.

The analysis of FLU, MBZ and their metabolites, previously described by Cvilink *et al*. (2008*a*), was slightly modified. The column Ascentis Express, 100 mm × 3 mm, 2·7 μm (Supelco – Sigma-Aldrich, Prague, Czech Republic) was used. The parent drugs FLU and MBZ were detected by UV/VIS photodiode array detector SPD-MD10Avp at 246 nm. The fluorescence detector (Shimadzu RF-10AXL, $\lambda_{EX} = 286$ nm, $\lambda_{Em} = 306$ nm) served for detection of FLU-R and MBZ-R. The mobile phase and the flow rate were the same as in the case of ABZ and its metabolites. Under these chromatographic conditions, the retention times were 5·6 min (FLU), 2·0 min (FLU-R), 4·4 min (MBZ) and 1·75 min (MBZ-R).

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Table 1. Specific activities of oxidation, reduction and conjugation enzymes tested in microsome-like, cytosol-like and mitochondria-like subcellular fractions of *Hymenolepis diminuta* homogenates

(The values represent means ± S.D. from 3–6 samples. CBR, carbonyl reductase; 11β-HSD, 11 β-hydroxysteroid dehydrogenase; 3α-HSD, 3α -hydroxysteroid dehydrogenase.)

Enzyme		Microsomes	Cytosol	Mitochondria
Peroxidase	(nmol·min^{-1}·mg^{-1})	1·95 ± 0·19	5·30 ± 0·21	0·36 ± 0·24
Catalase	(μmol·min^{-1}·mg^{-1})	5·41 ± 0·08	4·09 ± 0·12	5·19 ± 0·05
Superoxide dismutase	(nmol·min^{-1}·mg^{-1})	2·21 ± 0·25	3·93 ± 0·12	1·81 ± 0·40
Acenaphthenol dehydrogenase (AKR 1C)	(nmol·min^{-1}·mg^{-1})	n.d.	319·42 ± 1·80	n.d.
Menadione reductase (CBR)	(nmol·min^{-1}·mg^{-1})	n.d.	53·68 ± 7·11	n.d.
4-pyridinecarboxaldehyde reductase (AKR 1C, 1A1, 3α-HSD)	(nmol·min^{-1}·mg^{-1})	61·32 ± 12·76	40·11 ± 8·36	n.d.
Daunorubicin reductase pH 8·5 (AKR 1A)	(nmol·min^{-1}·mg^{-1})	n.d.	3·95 ± 0·70	n.d.
Oracin reductase (AKR 1C, 1B10, 11β-HSD 1)	(nmol·min^{-1}·mg^{-1})	n.d.	0·054 ± 0·05	n.d.
UDP-glucosyl transferase	(nmol·min^{-1}·mg^{-1})	n.d.	n.d.	1·93 ± 0·26
Glutathione S-transferase	(nmol·min^{-1}·mg^{-1})	n.d.	223·06 ± 36·16	n.d.

n.d., not detected.

Liquid chromatography/mass spectrometry

SPE and sample preparation. Samples analysed by LC/MS were comprised of samples incubated *in vitro* and *ex vivo* at a concentration of 10 μM of parent drugs. Samples were extracted using 2 SPE methods, previously described by Cvilink *et al*. (2009*c*).

LC/MS. The LC system comprised a Surveyor MS pump and a Surveyor autosampler (both ThermoFinnigan, San Jose, CA, USA). The column employed was a SymmetryShield RP18 (2·1 × 100 mm, 3·5 μm; Waters, Milford, USA). The measurements in positive and negative ion mode were carried out according to the protocol of Cvilink *et al*. (2009*c*) with minor changes of mode values. The following optimized ion source parameters were set for BZD drug analysis during sample data acquisition in positive ion mode (negative ion mode values in parentheses): spray voltage, 5·3 kV (−3·6 kV); capillary voltage, 3·0 V (−7·0 V); heated capillary temperature, 200 °C; tube lens offset voltage, 40·0 V (−15·0 V); sheath gas flow rate, 20·0 arbitrary units; auxiliary gas flow rate, 10·0 arbitrary units.

Ion optics settings were as follows: multipole 1 offset voltage, −2·0 V (0·5 V); lens voltage, −16·0 V (18·0 V); multipole 2 offset voltage, −6·5·0 V (10·0 V); multipole RF amplitude, 550 V (400 V). Nitrogen was used as both sheath and auxiliary gas and helium was used as the damping gas. Data acquisition and processing were carried out using Xcalibur software (version 1.2).

Fragmentation of parent BZD drugs and screening for metabolites

All BZD drug working standard solutions were introduced into the mass spectrometer at a flow rate of 5 μl min^{-1} using the inbuilt syringe pump. For more details about preparation of BZD drug standard solutions, see Cvilink *et al*. (2009*c*).

The spectra of BZD drugs were recorded over the mass range of *m/z* 50–650 in MS experiments; for MS^n experiments the range was variable. The MS and MS^n spectra were measured in order to obtain fragmentation patterns of all parent compounds and thus facilitate the spectral interpretation of novel unknown metabolites.

The screening for metabolites was carried out according to the protocol of Cvilink *et al*. (2009*c*). The structures of metabolites were tentatively assessed by the combination of their corresponding full-scan spectra, MS^n spectra and chromatographic behaviour.

Statistical analysis

The reported data are expressed as mean ± S.D. Statistical comparisons were carried out using Student's *t*-test (Microsoft Office Excel 2007). A probability of $P < 0{\cdot}05$ was considered statistically significant.

RESULTS

Enzyme assays

The activities of biotransformation enzymes were tested in microsome-like, cytosol-like and mitochondria-like fractions of *H. diminuta* homogenates. The methods based on spectrophotometric, spectrofluorometric and HPLC evaluation of the rising product of biotransformation or the diminishing substrate/cofactor were used. The resulting data expressed as specific activities are summarized in Table 1. In *H. diminuta* fractions, the activities of almost all assayed enzymes were detected. The activities of oxidation enzymes (Px, CAT, SOD) were found in all fractions, whereas the activity of conjugation enzymes was found only in the cytosol (GST) and mitochondria (UGlcT). Regarding

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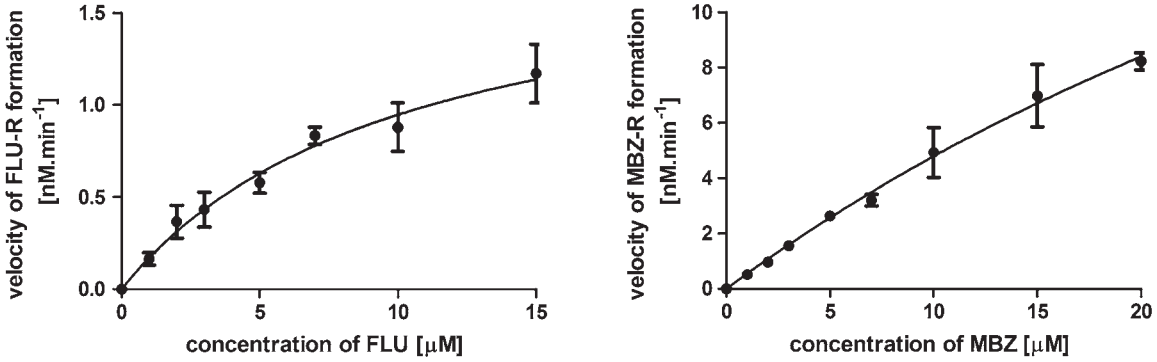

Fig. 1. Kinetics of flubendazole (FLU) and mebendazole (MBZ) reduction in cytosol-like fraction from *Hymenolepis diminuta* homogenate.

reduction enzymes, the cytosol-like fraction reduced acenaphthenol, menadione, daunorubicin at pH 8·5, oracin and 4-pyridinecarboxaldehyde. The last-mentioned substrate was also metabolized by a microsomes-like fraction. The UGT catalysing glucuronidation of p-nitrophenol and reduction activity towards metyrapone and daunorubicin at pH 6·0 failed to show any activity in all fractions.

HPLC analysis of ABZ, ABZ.SO, FLU and MBZ biotransformation in vitro

All fractions of *H. diminuta* homogenate were separately incubated with the anthelmintics ABZ, ABZ.SO, FLU or MBZ and co-enzyme NADPH. Using ABZ, no metabolites were formed in any incubation. To determine if either the second step of ABZ oxidation (formation of ABZ.SO_2 from ABZ.SO) or the sulphoreduction of ABZ.SO back to ABZ were occurring, we performed incubation with ABZ.SO as a substrate. Neither of these reactions was observed. In the case of FLU and MBZ, only metabolites with a reduced carbonyl group (FLU-R, MBZ-R) were found in the cytosol-like fraction, whereas microsome-like and mitochondria-like fractions showed no FLU or MBZ metabolites. Comparing FLU and MBZ, the *in vitro* reduction of MBZ was approximately 3·2 and 5·6 times more intensive than a reduction of FLU using, respectively, 1·0 and 10·0 μM concentrations of parent drugs.

Kinetics of FLU and MBZ reduction in vitro

The cytosol-like fraction of *H. diminuta* homogenate was incubated with various concentrations of FLU (1·0–15·0 μM) and MBZ (1·0–20·0 μM). The amount of FLU-R and MBZ-R formed in the incubation mixture was expressed as a reaction velocity. The direct plot of the reaction velocity versus substrate concentration fit well the Michaelis–Menten equation, and is shown for both drugs in Fig. 1. Using GraphPad Prism 5.0 software, the values of the basic kinetic parameters, apparent maximal velocity V'_{max} and apparent Michaelis constant K'_m, were calculated. The apparent maximal velocity V'_{max} indicates the rate of product formation at enzyme saturation with the substrate. The apparent Michaelis constant K'_m expresses the affinity of enzymes toward the substrate in the multi-enzymatic system. The ratio of these kinetic parameters (V'_{max}/K'_m) represents enzymatic efficiency, the so-called intrinsic clearance. The values of the kinetic parameters are recorded in Table 2. The apparent maximal velocity V'_{max} and apparent Michaelis constant K'_m for MBZ were approximately 18 times and 6 times higher than those for FLU.

Table 2. The kinetic parameters obtained for FLU-R and MBZ-R formation analysed by HPLC after a 30 min incubation of cytosol-like fraction with various concentrations of FLU or MBZ

(The results represent means ± S.D. from 4 samples. V'_{max}, apparent maximal velocity of the reaction; K'_m, apparent Michaelis constant; Cl_{int}, intrinsic clearance defined as V'_{max}/K'_m ratio; FLU, flubendazole; FLU-R, reduced flubendazole; MBZ, mebendazole; MBZ-R, reduced mebendazole.)

	V'_{max} (nM·min^{-1})	K'_m (μM)	Cl_{int} (min^{-1})
FLU-R formation	1·9 ± 0·2	10·3 ± 2·1	0·00018
MBZ-R formation	34·1 ± 6·7	61·3 ± 14·9	0·00056

HPLC analysis of ABZ, FLU and MBZ biotransformation ex vivo

The living tapeworms were cultivated individually in plastic flasks in 10 ml of RPMI 1640 medium containing ABZ, FLU or MBZ (1·0 μM and 10·0 μM) for 24 h. To evaluate the viability of the tapeworms, the motility was visually observed over an experiment period as a body movement which is caused mainly by rhythmical contractions of longitudinal musculature in strobila. Although the motility diminished during the incubation, all tapeworms showed noticeable undulating movement of their bodies after 24 h. The analyses of metabolites were carried out in a cultivation medium and homogenate

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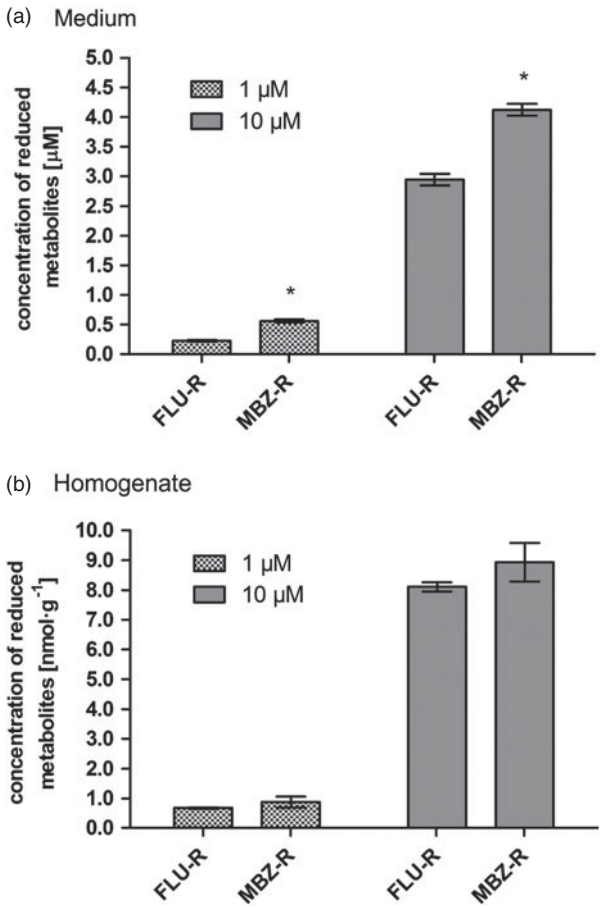

Fig. 2. Concentration of reduced metabolites (FLU-R, MBZ-R) analysed by HPLC after 24-h incubation of living tapeworms with 1 μM or 10 μM FLU or MBZ. (a) In incubation medium (μM). (b) In homogenate of parasites' bodies (nmol per g of body weight) Results represent means ± S.D., $n = 3$. * Significantly different compared with FLU-R, $P < 0{\cdot}05$.

of tapeworms' bodies, which had previously been rinsed with phosphate-buffered saline. As in the experiments performed *in vitro*, ABZ did not undergo any biotransformation in living tapeworms. The reduced metabolites FLU-R and MBZ-R were the only Phase I metabolites detected after *ex vivo* incubation of the tapeworms with FLU and MBZ. In the medium, MBZ-R rose to a higher level than FLU-R in both concentrations of the parent drugs (2·5 times for 1 μM and 1·4 times for 10 μM substrate concentration). The amount of metabolites measured in the homogenate of parasites did not show a significant difference between FLU-R and MBZ-R. For the results of the *ex vivo* biotransformation, see Fig. 2.

LC-MS^n analysis of ABZ and ABZ.SO metabolites

ABZ, the parent drug, was observed at m/z 266 (elution time 12·6 min). No phase I or II metabolites were found during the analysis of the samples.

Protonated ABZ.SO, used as the parent drug, occurred at m/z 282. No phase I or phase II metabolites were found during the analysis of the samples. No sulpho-reduction of ABZ.SO to ABZ was observed.

LC-MS^n analysis of FLU metabolites

The only phase I metabolite of the parent drug FLU (elution time 14·4 min, m/z 314) detected in our experiments was FLU-R (m/z 316, eluting at 10·3 min). FLU-R was identified according to both the FLU-R standard and the metabolite MS/MS spectra, each of which featured the typical product 284. Also the retention times were the same.

Two ions at m/z 330 were detected eluting at 11·2 min (MFLU-R1) and 12·2 min (MFLU-R2). In both cases, MS/MS analyses revealed a ready loss of 32 u (methanol), which was typical for all BZD anthelmintics and signified a BZD origin. The m/z 330 would correspond to a methyl derivative of FLU-R. When compared with the fragmentation schemas and chromatographic behaviour for the methyl derivative of FLU-R published previously by Cvilink *et al*. (2009*c*), it can be assumed that these ions represent the same metabolites.

LC-MS^n analysis of MBZ metabolites

As MBZ is basically defluorinated FLU, similarities in fragmentation behaviour could be expected. The parent drug MBZ (m/z 296) eluted at 13·8 min. The major and the only phase I metabolite detected was MBZ-R at m/z 298, eluting at 9·6 min. The MS/MS spectra of MBZ-R contained the main ion at m/z 266 which, allowing for the absence of fluorine, fully corresponded to FLU-R MS/MS product ions. Identification of MBZ-R was confirmed by MS/MS analysis of the MBZ-R standard, which yielded identical MS/MS spectra, as well as by the identity of their retention times. No methyl derivate of MBZ-R or any other phase II metabolite was observed.

The retention times, m/z ratios and product ions of BZD metabolites detected in MS^n experiments are summarized in Table 3. All the measurements were carried out in the positive ion mode, as the signal was approximately 3 to 4-fold higher than in the negative ion mode. No anionic metabolites were observed when analysing the samples using the negative ion mode measurements. Structures of the parent anthelmintic drugs and the identified metabolites are recorded in Fig. 3.

DISCUSSION

H. diminuta is often used as a laboratory model for the study of the physiology, biochemistry and immunology of tapeworms (Muller and Wakelin, 2002; Horak and Klimes, 2007). The project presented here was focused on the activities of drug-metabolizing

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Table 3. MS^n spectra and retention times (R_t) of detected benzimidazole metabolites

Metabolite	R_t (min)	m/z	MS stage	Product ions (m/z, relative abundance %)	Collision energy (%)
FLU-R	10,2	316	MS^2	284 (100), 160 (1), 125 (1)	40
MFLU-R1	11,2	330	MS^2	298 (100)	40
		298	MS^3	97 (2), 123 (2), 125 (4), 174 (20), 280 (4), 298 (100)	40
MFLU-R2	12,2	330	MS^2	270 (100), 273 (6), 298 (62), 330 (15)	40
		298	MS^3	270 (100)	40
		270	MS^4	97 (16), 146 (87), 252 (16), 270 (100)	40
MBZ-R	9,6	298	MS^2	266 (100), 160 (1)	40

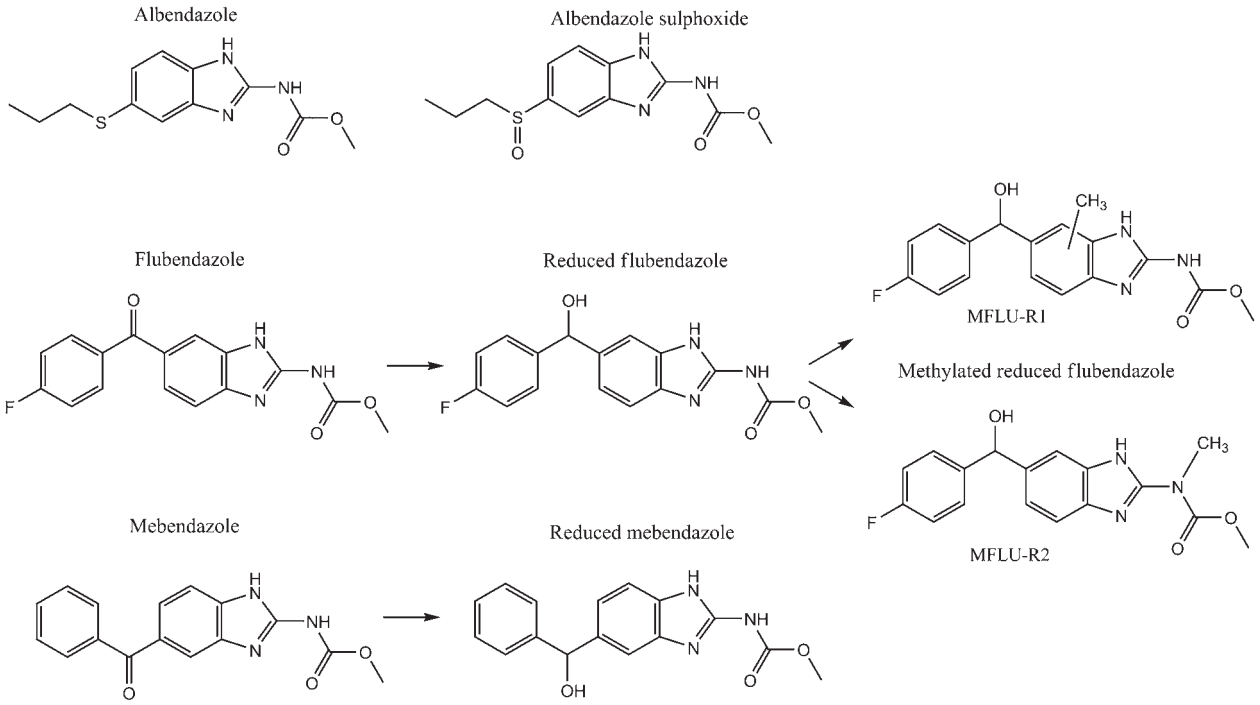

Fig. 3. Chemical structures of used anthelmintic drugs and identified metabolites.

enzymes towards some anthelmintics and other xenobiotics in this model tapeworm.

First, the reductive biotransformation was assayed, as reductive metabolism seems to be the main metabolic pathway of detoxification in helminths. This is understandable, since the intestinal lumen retains the low redox environment. Reduction is the major detoxification route of carbonyl group-bearing compounds (Munir and Barrett, 1985; Cvilink *et al*. 2009*a*). The enzymes involved in the reduction of aldehydes and ketones belong to 3 classes: short chain dehydrogenases, medium chain dehydrogenases and aldo-keto reductases (AKR). In our study, several model substrates (acenaphthenol, daunorubicin at pH 6·0 and 8·5, metyrapon, 4-pyridinecarboxaldehyde, oracin) were used in the determination of the activities of carbonyl-reducing enzymes. In *H. diminuta*, almost all of the above-mentioned model substrates (except metyrapone and daunorubicin at pH 6·0) underwent enzymatic reduction. The ability to metabolize these substrates as well as a variety of other compounds (Munir and Barrett, 1985) proves that *H. diminuta* effectively reduces aldehydes and ketones.

This conclusion is further supported by the results of our biotransformation study with FLU and MBZ. HPLC and LC/MS analyses showed that the carbonyl group of both anthelmintics was reduced. The same outcome with FLU and MBZ has also been identified in other parasitic species – *Haemonchus contortus* (Barber's pole worm) (Cvilink *et al*. 2008*a*,*b*), *Dicrocoelium dendriticum* (lancet fluke) (Moreno *et al*. 2004; Cvilink *et al*. 2009*c*) and *Moniezia expansa* (sheep tapeworm) (Moreno *et al*. 2004). Data obtained during *in vitro* experiments with *H. diminuta* also served for the evaluation of basic kinetic parameters for the FLU and MBZ reduction. Although the maximal velocity of MBZ reduction is nearly 18 times higher than FLU reduction, reductases possess a 6-times higher affinity for FLU. But generally these values indicate a slow reaction (velocity approximately $nM \cdot min^{-1}$) and a relatively low enzyme affinity to both drugs. In *H. contortus*, an approximately 20-times faster

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reduction rate of FLU and 7-times higher affinity of reductases to FLU was reported (Cvilink *et al*. 2008*a*).

The presence of drug-oxidation enzymes in helminths had not been proven for many years (Barrett, 1997, 1998). However, several studies conducted later showed evidence of drug-oxidation enzymes as well as the ability to oxidize certain xenobiotics in some helminth species. The activity of cytochrome P450 (CYP) was found in *Schistosoma mansoni* (blood fluke) (Saeed *et al*. 2002), in the larval stages of *H. contortus* (Kotze, 1997) and in murine intestinal nematode *Heligmosomoides polygyrus* (Kerboeuf *et al*. 1995). In addition to CYP, the oxidative metabolism of xenobiotics may also be mediated by enzymes, which primarily serve as a defence against oxidative stress and help to maintain redox balance in parasites, e.g. peroxidase (Px), glutathione peroxidase, superoxide dismutase (SOD), catalase (CAT), thioredoxin glutathione reductase, cytochrome c peroxidase, xanthine oxidase, and peroxiredoxins (Barrett, 1997; Henkle-Duhrsen and Kampkotter, 2001; Salinas *et al*. 2004; Kuntz *et al*. 2007; Cvilink *et al*. 2009*a*).

In our experiments with *H. diminuta*, significant activities of Px, SOD and CAT were found, but no oxidative metabolite of anthelmintic drug ABZ or ABZ.SO was detected by HPLC and LC/MS, neither after *in vitro* or *ex vivo* incubation. Contrary to these results, another tapeworm, *M. expansa*, metabolized ABZ via the oxidative pathway during *in vitro* experiments (Barrett, 1997; Solana *et al*. 2001). Also many helminths from other classes, e.g. *H. contortus* (Cvilink *et al*. 2008*b*), *Ascaris suum* (large roundworm of pigs), *Fasciola hepatica* (liver fluke) (Solana *et al*. 2001) and *D. dendriticum* (Cvilink *et al*. 2009*b*; Bartikova *et al*. 2010*b*), were able to oxidize ABZ to ABZ.SO. Further oxidation of ABZ.SO to its sulphone has been observed in *H. contortus*, *D. dendriticum* and *F. hepatica*, but this has so far not been found in any cestode. Very intensive sulphonation was reported in the case of triclabendazole in *F. hepatica* (Robinson *et al*. 2004). In the ovine tapeworm *M. expansa*, ABZ.SO undergoes sulpho-reduction to ABZ. However, in our experiments no sulpho-reduction of ABZ.SO was observed in *H. diminuta*. Also *F. hepatica* and *D. dendriticum* have been shown not to possess any sulpho-reduction ability (Solana *et al*. 2001; Robinson *et al*. 2004; Cvilink *et al*. 2009*b*). These results demonstrate that the metabolic pathway of ABZ varies significantly among helminths, and even within the group of tapeworms themselves.

Phase II of drug biotransformation corresponds to conjugation reactions of the drug or other substrate with certain endogenous compounds. Glutathione S-transferases (GST) are the most important conjugation enzymes which protect organisms against reactive electrophilic compounds. In *H. diminuta*, significant activity of GST was found in the cytosol-like fraction. This activity was comparable with that reported in sheep (Bartikova *et al*. 2010*a*). Although the activity of GST in *H. diminuta* was high, no glutathione conjugates with the anthelmintics were detected. This result is in accordance with previous findings which have indicated that common anthelmintics appear not to be the suitable substrates of the helminth's GST (Cvilink *et al*. 2009*a*).

Other conjugation enzymes, UDP-glucosyl transferases, showed a weak activity only in the mitochondria-like fraction and the activity of UDP-glucuronosyl transferase was not observed in any fraction. Also no formation of glucoside or glucuronide from the anthelmintics was found. On the other hand, *H. contortus* uses glucose conjugation to deal with FLU, FLU-R and ABZ (Cvilink *et al*. 2008*b*). Glucose conjugation as a detoxification mechanism has been further observed in *A. suum* and *Parascaris equorum* (horse roundworm) (Cvilink *et al*. 2009*a*). Although this metabolic pathway occurs rarely in the animal kingdom, it seems to be the main conjugation pathway of some parasitic species (Cvilink *et al*. 2008*b*).

Methylation is the further phase II biotransformation pathway described in helminths (Cvilink *et al*. 2009*c*). Although methylation of anthelmintics ABZ, MBZ or FLU has not been reported in mammals, a biotransformation study of FLU in *H. diminuta* revealed the formation of methyl derivatives of FLU-R. Two monomethyl derivatives with methyl groups in different positions – one on the aromatic ring, the other on the side chain of FLU-R – were identified. This shows that *H. diminuta* is probably capable of synthesizing S-adenosylmethionine, an essential co-factor of methylation in mammals. Despite the other anthelmintics and their metabolites tested, FLU-R seems to be the only suitable substrate for methyltransferases in *H. diminuta*, as no methyl derivatives of MBZ, MBZ-R or ABZ were detected. This result suggests the high substrate selectivity of methyltransferases in *H. diminuta*. Methylation as a metabolic pathway in helminths was reported for the first time in *D. dendriticum* by Cvilink *et al*. (2009*c*). But unlike *H. diminuta*, this parasite also methylated MBZ-R.

Regarding biotransformation of non-BZD drugs in *H. diminuta*, detailed knowledge about this phenomenon is not available so far. However, one of these drugs, a pyrazinoisoquinoline drug praziquantel, has been tested recently. No Phase I or Phase II metabolite of praziquantel was detected in *ex vivo* or *in vitro* experiments using ultra high performance liquid chromatography/tandem mass spectrometry (*unpublished observations*). Other non-BZD drugs have not been studied to date.

The results of our project demonstrate that *H. diminuta* possesses a unique enzymatic system which is able to affect the structure of administered

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xenobiotics, thus protecting the parasites against their negative action. *H. diminuta* metabolizes selected anthelmintics by means of reduction (FLU, MBZ) and methylation (FLU), but not by oxidation (ABZ, ABZ.SO), nor by hydrolysis (FLU, MBZ). The detected biotransformation pathways of anthelmintics found in *H. diminuta* differ from those previously described in *M. expanza*, another tapeworm, or in other classes of helminths. These findings suggest that interspecies differences in drug metabolism among species of helminths also exist between close relatives. Therefore, *H. diminuta* is not an ideal model for the study of drug metabolism in the tapeworms of humans and ruminants.

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