The metabolic fate of ivermectin in host (*Ovis aries*) and parasite (*Haemonchus contortus*)

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SUMMARY

Ivermectin (IVE), one of the most important anthelmintics, is often used in the treatment of haemonchosis in ruminants. The objective of our work was (1) to find and identify phase I and II metabolites of IVE formed by the Barber's pole worm (*Haemonchus contortus*), and (2) to compare IVE metabolites in helminths with IVE biotransformation in sheep (*Ovis aries*) as host species. Ultrahigh-performance liquid chromatography/tandem mass spectrometry (UHPLC/MS/MS) was used for this purpose. During *in vitro* incubations, microsomes (from adult worms or from ovine liver) and a primary culture of ovine hepatocytes were incubated with IVE. In the *ex vivo* study, living *H. contortus* adults were incubated in the presence of 1 μM IVE for 24 h. The results showed that the *H. contortus* enzymatic system is not able to metabolize IVE. On the other hand, 7 different phase I as well as 9 phase II IVE metabolites were detected in ovine samples using UHPLC/MS/MS analyses. Most of these metabolites have not been described before. *Haemonchus contortus* is not able to deactivate IVE through biotransformation; therefore, biotransformation does not contribute to the development of IVE-resistance in the Barber's pole worm.

Key words: ivermectin, *Haemonchus contortus*, biotransformation, UHPLC/MS/MS.

INTRODUCTION

The Barber's pole worm (*Haemonchus contortus*) is a very common gastrointestinal nematode and one of the most pathogenic parasites of ruminants (e.g., sheep and goats). The control of haemonchosis has been and still is based on the use of anthelmintics. The infection is usually treated with broad-spectrum anthelmintics, such as benzimidazoles, macrocyclic lactones and salicylanilides (Getachew *et al*. 2007). However, the widespread and indiscriminate use of these treatments has led to the emergence of parasitic isolates with anthelmintic resistance to the main anti-parasitic drug groups. Several patterns of drug resistance have been described in helminths (i.e., Wolstenholme *et al*. 2004; James *et al*. 2009). One of these is associated with biotransformation enzymes that are responsible in some cases for the faster deactivation of anthelmintics in resistant parasites (Robinson *et al*. 2004; Alvarez *et al*. 2005; Devine *et al*. 2010*a*,*b*).

The biotransformation of benzimidazole anthelminthics has been studied and proven 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*. 2008*a*, *b*, 2009*a*,*b*). On the other hand, *H. contortus* was neither *in vitro* nor *ex vivo* able to metabolize the salicylanilide anthelmintic closantel (Rothwell and Sangster, 1997). The macrocyclic lactones (avermectins and milbemycins) are products (or chemical derivatives thereof) of soil microorganisms belonging to the genus *Streptomyces*. Alvinerie *et al*. (2001) have reported the formation of one metabolite of moxidectin in *H. contortus* homogenate incubations. The metabolic pathway of ivermectin (IVE) has been studied in mammals, with members of the cytochrome P450 superfamily identified as the principal metabolizing enzymes (Miwa *et al*. 1982; Chiu *et al*. 1984; Zeng *et al*. 1998). No information about IVE biotransformation in helminths has been made available so far.

The goal of the present work is to determine whether or not *H. contortus* is able to metabolize IVE. The metabolites of phase I and phase II IVE biotransformation formed by *H. contortus* were searched for *in vitro* as well as *ex vivo*. IVE biotransformation was also tested in sheep to compare the IVE metabolites formed in helminths with those formed in their hosts.

MATERIALS AND METHODS

Chemicals

Ivermectin H_2B_{1a} was purchased from Sigma–Aldrich (St Louis, MO, USA). Acetonitrile (LC/MS

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grade) and ammonium acetate were purchased from Sigma-Aldrich (St Louis, MO, USA). De-ionized water was prepared with the Demiwa 5-roi purification system (Watek, Ledeč nad Sázavou, Czech Republic). Liquid sterile-filtered RPMI-1640 medium, HAM F12 medium, Williams' E medium, foetal calf serum and all other chemicals (LC/MS, HPLC or analytical grade) were obtained from Sigma–Aldrich (Prague, Czech Republic).

Collection of parasite material

The susceptible isolate of *Haemonchus contortus* has 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. Four parasite-free lambs (3–4 months old) were orally infected with 5000 L3 larvae 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 from sheep abomasum using the agar method described by Van Wyk *et al*. (1980). The isolated parasites were either used immediately for *ex vivo* experiments or frozen at −80 °C for preparation of subcellular fractions for *in vitro* studies.

Preparation of microsomes and incubation with ivermectin (IVE)

Microsomes from homogenates of *H. contortus* adults were prepared as described previously (Cvilink *et al*. 2008*a*). Microsomes from the ovine liver were prepared by the same procedure. All microsomal fractions were stored at −80 °C. No other subcellular fractions (cytosolic or mitochondrial) were used for incubations with IVE. Protein concentrations were assayed using the bicinchoninic acid method according to the Sigma protocol.

The reaction mixture (total volume of 0·3 ml) contained 100 μl of microsomes (approximately 0·4 mg of proteins), 100 μM IVE pre-dissolved in dimethyl sulfoxide (concentration of DMSO in the reaction mixture was 1%), 1 mM NADPH and 0·1 M sodium phosphate buffer (pH 7.4). Blank samples contained 100 μl of 0·1 M sodium phosphate buffer instead of microsomes or 1% DMSO instead of IVE. All incubations were carried out at 37 °C for 30 min.

At the end of the incubation, 30 μl of ammonium hydroxide solution (25% v/v) and 700 μl of ethyl acetate were added. After shaking (3 min, vortex) and centrifugation (3 min, 5000 ***g***) of the mixture, the supernatants were removed and subsequently evaporated to dryness using vacuum concentrator. Samples were stored at −80 °C until LC/MS analysis.

Ex vivo *experiment*

Living nematodes were cultivated as described by Kotze and McClure (2001) with a 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 IVE was added. IVE was pre-dissolved in DMSO; the final concentration of DMSO in medium was 0·1%. Nematodes were incubated in medium with 1 μM IVE for 24 h. In chemical blank samples, medium was incubated with 1 μM IVE but without nematodes. In biological blank samples, nematodes were incubated with DMSO instead of IVE. After the incubation, medium was placed into the plastic tubes. Nematodes were repeatedly washed with phosphate buffer and transferred into the plastic tubes. Samples were frozen and stored under −80 °C. Prior to the analysis, parasite bodies were homogenized in redistilled water at the ratio of 1:3 (w/v) using Sonopuls (Bandelin, Germany). Medium and the parasite homogenate were then extracted using solid-phase extraction. Dried extracts were stored (− 80 °C) until UHPLC/MS analyses.

Isolation of hepatocytes and incubation of hepatocytes primary culture with IVE

Ovine hepatocytes were obtained from the ovine liver by a two-step collagenase method (Berry *et al*. 1991; Baliharová *et al*. 2004). Three million viable (80%) cells in 3 ml of culture medium ISOM (1:1 mixture of Ham F12 and Williams' E) were placed into 60 mm plastic dishes pre-coated with collagen. The fetal calf serum was added in culture medium (5%). Cultures were maintained without substrates for 4 h at 37 °C in a humid atmosphere of air and 5% CO_2. After attachment of hepatocytes, the ISOM medium was replaced with fresh serum-free medium with 10 μM IVE pre-dissolved in DMSO. The concentration of DMSO in the medium was 0·1% (v/v). Hepatocytes were incubated with IVE for 24 h at 37 °C in the humid atmosphere of air and 5% CO_2. At the end of the experiment, hepatocytes were scraped off into the incubation medium and homogenized using Sonopuls (Bandelin, Germany). Samples were then extracted using solid-phase extraction. Dried extracts were stored (− 80 °C) until UHPLC/MS analyses.

Solid-phase extraction and sample preparation

Samples from *ex vivo* experiments and hepatocyte incubations were extracted using solid-phase extraction. Two ml of the medium, parasite or hepatocyte homogenate were centrifuged at 3000 ***g*** for 5 min. The supernatant was loaded onto a Waters Oasis HLB extraction cartridge (1cc, 30 mg, 30 μm particles; Waters) previously conditioned by washing

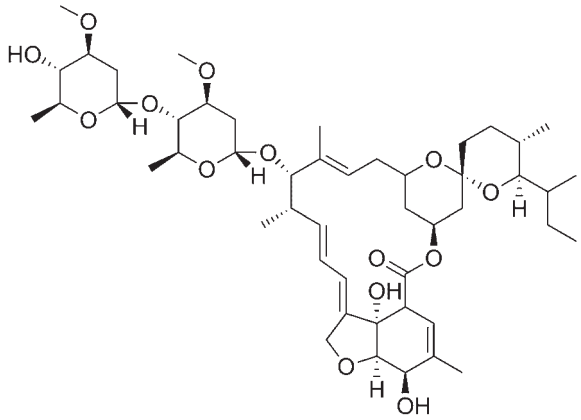

Fig. 1. Chemical formula of ivermectin H_2B_{1a} (IVE). $C_{48}H_{74}O_{14}$, exact mass 874·5079.

with 1 ml of acetone, 1 ml of methanol and 1 ml of redistilled water. In the next step, the cartridge was washed with 1 ml of 5% aqueous methanol (v/v). Compounds of interest were eluted with 1 ml of methanol followed by 1 ml of acetone. Eluates were evaporated to dryness using the vacuum concentrator Eppendorf 5310 (Hamburg, Germany) and stored at −80 °C until UHPLC/MS analyses.

Ultrahigh-performance liquid chromatography/tandem mass spectrometry (UHPLC/MS/MS)

Selected samples were quantitatively dissolved in 200 µl of a mixture of acetonitrile/water (1:1, v/v). UHPLC/MS/MS chromatograms of samples were measured in both positive and negative polarity modes using electrospray ionization (ESI) and hybrid quadrupole-time-of-flight (Q-TOF) Mass Analyzer microOTOF-Q (Bruker Daltonics, Bremen, Germany). UHPLC was performed on Agilent 1290 Infinity Liquid Chromatograph (Agilent Technologies, Waldbronn, Germany) using a Kinetex C18 column (150 mm × 2·1 mm, 1·7 µm, Phenomenex, Torrance, CA, USA), a temperature of 25 °C, a flow rate of 0·3 ml/min and an injection volume of 1 µl. The mobile phase consisted of acetonitrile (A) and 5 mM ammonium acetate buffer with pH 6.5 (B). The linear gradient was as follows: 0 min – 15% A, 15 min – 95% A, 17 min – 95% A; and finally washing and reconditioning of the column for 20 min. The Q-TOF mass spectrometer was used with the following setting of tuning parameters: capillary voltage 4·5 kV, drying temperature 200 °C, the flow rate and pressure of nitrogen were 7 l/min and 1 bar, respectively. The external calibration was performed with sodium formate clusters before individual measurements. ESI mass spectra were recorded in the range of m/z 50–1200 both in positive- and negative-ion modes. The isolation width $\Delta m/z$ 4 and the collision energy 20 eV using argon as the collision gas were used for MS/MS experiments. Advanced software tools, Metabolite Predict and Metabolite Detect (Bruker Daltonics, Bremen, Germany), were used for the data evaluation.

RESULTS AND DISCUSSION

In the present project the biotransformation of IVE (Fig. 1) was studied in the Barber's pole worm (*Haemonchus contortus*). All *in vitro* and *ex vivo* methods applied for this purpose have been used successfully in our previous studies, leading to the identification of several new metabolites of benzimidazole anthelmintics in helminths (Cvilink *et al.* 2008*a*,*b*).

The analytical characterization of IVE biotransformation samples was performed using UHPLC/MS/MS. First, full-scan and tandem mass spectra of IVE standard were measured and interpreted both in positive-ion and negative-ion modes of ESI (see Materials and Methods). ESI was chosen as the method of choice for the subsequent analysis of metabolic samples because of the expected formation of phase II metabolites. Ammonium adducts $[M+NH_4]^+$ and adducts with alkali metal ions, such as $[M+Na]^+$ and $[M+K]^+$, were observed in full scan positive-ion ESI mass spectra, while deprotonated molecules $[M-H]^-$ together with acetate adducts $[M+CH_3COO]^-$ were the most important ions in the negative-ion mode. Moreover, fragment ions observed mainly in positive-ion full-scan mass spectra helped to confirm the presence of IVE-related compounds. The accurate calibration of the mass scale enabled the mass accuracy, usually better than 5 ppm, to be achieved, resulting in the elemental composition determination of observed ions (Tables 1 and 2). In a few cases, mass accuracies were slightly worse than 5 ppm due to the very low abundances of these ions. The additional information was obtained using tandem mass spectra measurements in which typical neutral losses supported the identification of the metabolic reactions (Holčapek *et al.* 2010). Reconstructed ion current chromatograms and constant neutral loss chromatograms were used for a better visualization of the UHPLC/MS/MS chromatograms. The software tools *Metabolite Predict* and *Metabolite Detect*, already described in our previous paper (Jirásko *et al.* 2010), were applied for the prediction of metabolites and their subsequent detection. This software was set to predict 3 generations of possible IVE metabolite structures in accordance with all metabolic rules (Holčapek *et al.* 2008). The created list of particular exact m/z values was subsequently used in the process of detection of the metabolites, including the subtraction of chromatograms of placebo experiments from the chromatograms of the biotransformation samples (Fig. 2). As a result, difference chromatograms providing information about the presence of $[M+NH_4]^+$ or

Table 1. Phase I metabolites of ivermectin (IVE) in sheep samples detected by UHPLC/MS/MS

	Theoretical *m/z*			Mass accuracy [ppm]			
				Hepatocytes		Microsomes	
t_R [min]	$[M+NH_4]^+$	$[M-H]^-$	Metabolic reaction (elemental composition change)	$[M+NH_4]^+$	$[M-H]^-$	$[M+NH_4]^+$	$[M-H]^-$
8·5	894·5209	875·4798	$+O, -CH_2$	−3·1	−1·1	n.d.	n.d.
8·6	894·5209	875·4798	$+O, -CH_2$	−2·7	−1·1	n.d.	n.d.
9·0	894·5209	875·4798	$+O, -CH_2$	n.d.	n.d.	−1·1	−6·2
9·6	908·5365	889·4954	$+O$	−2·0	−1·2	−2·5	−5·0
9·8	908·5365	889·4954	$+O$	2·2	−4·6	6·9	−4·9
14·0	908·5365	889·4954	$+O$	n.d.	n.d.	−2·6	−3·3
14·1	878·5260	859·4849	$-CH_2$	0·3	1·2	−4·0	2·6
15·5	892·5416	873·5005	Parent drug (IVE)	−2·0	−1·2	2·2	−1·9

t_R, Retention time.
Metabolic reaction, description of elemental composition change – demethylation $-CH_2$, hydroxylation $+O$, sulfation $+SO_3$, glucuronidation $+C_6H_8O_6$.
Mass accuracy, particular mass accuracies of experimental *m/z* of $[M+NH_4]^+$ and $[M-H]^-$.
n.d., Not detected.

Table 2. Phase II metabolites of ivermectin (IVE) in sheep samples detected by UHPLC/MS/MS

	Theoretical *m/z*			Mass accuracy [ppm]			
				Hepatocytes		Microsomes	
t_R [min]	$[M+NH_4]^+$	$[M-H]^-$	Metabolic reaction (elemental composition change)	$[M+NH_4]^+$	$[M-H]^-$	$[M+NH_4]^+$	$[M-H]^-$
4·7	990·4726	971·4315	$+2O, -CH_2, +SO_3$	6·3	5·4	n.d.	n.d.
5·1	1004·4883	985·4472	$+2O, +SO_3$	n.d.	−3·7	n.d.	n.d.
5·2	988·4934	969·4523	$+O, +SO_3$	n.d.	−0·6	n.d.	n.d.
5·2	960·4621	941·4210	$+O, -2CH_2, +SO_3$	n.d.	0·8	n.d.	n.d.
5·4	974·4777	955·4366	$+O, -CH_2, +SO_3$	10	2·1	n.d.	n.d.
5·7	974·4777	955·4366	$+O, -CH_2, +SO_3$	n.d.	0·1	n.d.	n.d.
5·9	988·4934	969·4523	$+O, +SO_3$	−5·8	2·8	n.d.	n.d.
6·0	1070·5530	1051·5119	$+O, -CH_2, +C_6H_8O_6$	1·4	1·1	n.d.	n.d.
6·1	990·4726	971·4315	$+2O, -CH_2, +SO_3$	n.d.	−2·3	n.d.	n.d.
15·5	892·5416	873·5005	Parent drug (IVE)	−2·0	−1·2	2·2	−1·9

t_R, Retention time.
Metabolic reaction, description of elemental composition change – demethylation $-CH_2$, hydroxylation $+O$, sulfation $+SO_3$, glucuronidation $+C_6H_8O_6$.
Mass accuracy, particular mass accuracies of experimental *m/z* of $[M+NH_4]^+$ and $[M-H]^-$.
n.d., Not detected.

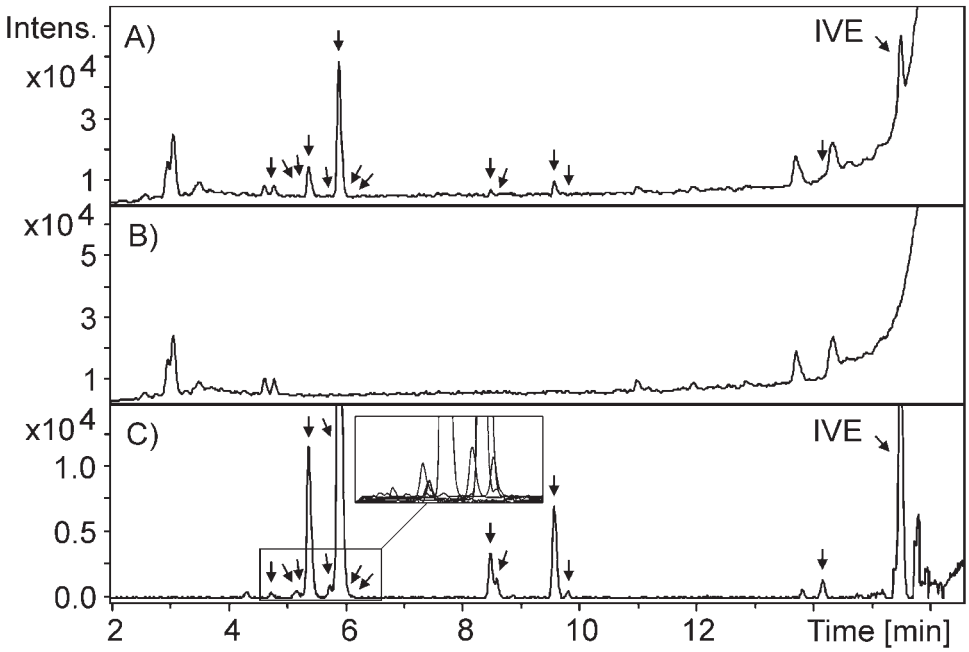

Fig. 2. Chromatogram subtraction of the placebo experiment from the ivermectin (IVE) biotransformation sample using *Metabolite Detect* software in the negative polarity mode of ESI. (A) Total ion current chromatogram of the sheep hepatocytes extract. (B) Total ion current chromatogram of the placebo sample. (C) Difference chromatogram with insert zoom of extracted ion chromatograms of individual metabolites (presented in Table 2). Arrows show detected metabolites (signal/noise $\geqslant 5$).

$[M-H]^-$ ions of individual drug metabolites were generated.

To begin with, phase I metabolites of IVE in *H. contortus* were searched for in an IVE incubation of subcellular fractions of the worms. The microsome-like fraction from *H. contortus* was incubated with 100 μM IVE. In all samples, only ammonium adducts of the IVE molecule at *m/z* 892·5416 in the positive-ion mode and the deprotonated IVE molecule at *m/z* 873·5005 in the negative-ion mode at the retention time of 15·5 min were observed, and no phase I metabolites produced by the Barber's pole worm were detected.

Consequently, *ex vivo* incubations of 1 μM IVE were done with living helminths previously isolated from their hosts. Lower concentrations of IVE in the *ex vivo* study than in the *in vitro* experiments were used to avoid death of the worms and also to approximate incubation conditions to real plasmatic concentration of IVE in animals. In a medium at an IVE concentration up to 1 μM, the movement of helminths was visible during the entire incubation period and thus the helminths were alive during the whole experiment. After 24-hour incubations, medium and worm homogenates were analysed by the UHPLC/MS/MS technique and both phase I and II metabolites of IVE were searched for. In all samples, only IVE was detected without any trace of IVE metabolites.

On the other hand, when the biotransformation of benzimidazole anthelmintics was studied in *H. contortus*, several metabolites were found and identified *in vitro* as well as *ex vivo* (Cvilink *et al.* 2008*a*,*b*). *Haemonchus contortus* can metabolize albendazole via sulphoxidation and glucose conjugation, and flubendazole via the reduction of a carbonyl group and subsequent glucose conjugation (Cvilink *et al.* 2008*b*). The negative results in the detection of IVE metabolites in *H. contortus* necessitate additional confirmation that our analytical method was well optimized and capable of the highly sensitive detection of all possible IVE metabolites in the studied samples. For this reason, we decided to study the biotransformation of IVE in sheep *in vitro* at both the subcellular and cellular levels. Ovine liver microsomal fractions were incubated with 100 μM IVE, and primary cultures of ovine isolated hepatocytes were incubated with 10 μM IVE. The samples obtained were analysed using UHPLC/MS/MS. In the ovine samples, numerous IVE metabolites were detected. The phase I metabolites correspond to the IVE hydroxylation (+O), demethylation (-CH_2) and the combination of both processes. The conjugation with glucuronic and sulphuric acids represented the phase II of the IVE biotransformation. In total, 7 different phase I (Table 1) and 9 phase II (Table 2) metabolites were identified using UHPLC/MS/MS analyses. These results clearly demonstrate the high sensitivity of our methods, resulting in the detection of new IVE metabolites in sheep not reported so far.

Previous metabolic studies of IVE performed in rats, cattle, sheep, goats, and pigs have revealed only

Phase I metabolites: 24-OH-H_2B_{1a} and 24-OH-H_2B_{1b} in cattle, sheep, and rats (Chiu *et al*. 1986), 3″-*O*-desmethyl-H_2B_{1a} and 3″-*O*-desmethyl-H_2B_{1b} in pigs and goats (González Canga *et al*. 2009). No phase II metabolites have been reported so far. In our experiments, 9 different phase II metabolites of IVE were found in incubations with primary cultures of ovine hepatocytes; 8 conjugates with sulphuric acid and 1 with glucuronic acid.

The present study was designed to advance our knowledge about the metabolism of IVE in helminths and their hosts. Despite the highly sensitive UHPLC/MS/MS analyses, no IVE metabolite formed in the Barber's pole worm (*H. contortus*) was detected. This finding indicates that this nematode is not able to deactivate IVE through biotransformation; therefore, biotransformation does not contribute to the development of IVE-resistance in the Barber's pole worm. In sheep, the host organism of *H. contortus,* the UHPLC/MS/MS technique allowed us to find and identify 16 different IVE metabolites, most of which had not yet been described.

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