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The induction and inhibition of UDP-glycosyltransferases in *Haemonchus contortus* and their role in the metabolism of albendazole

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ABSTRACT

Albendazole (ABZ) is an anthelmintic frequently used to treat haemonchosis, a common parasitosis of ruminants caused by the gastrointestinal nematode *Haemonchus contortus*. This parasite is able to protect itself against ABZ via the formation of inactive ABZ-glycosides. The present study was designed to deepen the knowledge about the role of UDP-glycosyltransferases (UGTs) in ABZ glycosylation in *H. contortus*. The induction effect of phenobarbital, a classical inducer of UGTs, as well as ABZ and ABZ-sulphoxide (ABZSO, the main active metabolite of ABZ) on UGTs expression and UGT activity toward ABZ was studied *ex vivo* in isolated adult nematodes. The effect of three potential UGT inhibitors (5-nitrouracil, 4,6-dihydroxy-5-nitropyrimidine and sulfinpyrazone) on ABZ glycosylation was tested. Pre-incubation of nematodes with ABZ and ABZSO led to increased expression of several UGTs as well as ABZ-glycosides formation in subsequent treatment. Phenobarbital also induced UGTs expression, but did not affect ABZ biotransformation. In the nematode's subcellular fraction, sulfinpyrazone inhibited UGT activity toward ABZ, although no effect of other inhibitors was observed. The inhibitory potential of sulfinpyrazone on the formation of ABZ-glycosides was also proved *ex vivo* in living nematodes. The obtained results confirmed the role of UGTs in ABZ biotransformation in *H. contortus* adults and revealed sulfinpyrazone as a potent inhibitor of ABZ glycosylation in this parasite. The possible use of sulfinpyrazone with ABZ in combination therapy merits further research.

1. Introduction

Haemonchus contortus is one of the most economically important gastrointestinal parasitic nematodes of grazing ruminants, contributing to the reduction or loss of livestock production (Besier et al., 2016). The control of this parasite mainly relies on the use of anthelmintic drugs. Nevertheless, over the past few decades *H. contortus* has developed resistance to all major anthelmintic drug classes (benzimidazoles, imidazothiazoles, macrocyclic lactones, acetonitrile derivate) (Kotze and Prichard, 2016). Excessive and improper use of anthelmintics causes selective pressure on the survival of resistant worms. The great adaptability of *H. contortus* to environmental and host condition is supported by a high level of genetic diversity connected with high fecundity (the production of thousands of eggs per day) along with large infections of several thousand worms in host animals (Kaplan and Vidyashankar, 2012). Moreover, the ability to survive adverse conditions such as prolonged drought and cold winters leads to an extension to the geographical range within which nematode populations develop routinely, resulting in the widespread and growing problem of anthelmintic resistance (Besier et al., 2016; Salle et al., 2019). Among the approaches to delay the development of drug resistance is to use combinations of drugs from different chemical classes or drugs together with the bioactive phytochemicals supporting their anthelmintic activity. Improving nutritional status and the hosts' immune response against infection has also been used. Due to possible pharmacokinetics/dynamics interactions, however, the use of these combinations has the potential of promoting multidrug-resistant nematodes (Lanusse et al., 2018). Therefore, the discovery or development of new strategies to overcome anthelmintic resistance together with obtaining a deeper understanding of the mechanisms of resistance has come to the forefront of parasitological research.

The main mechanism of anthelmintic resistance is the target-site resistance, e.g. mutations in genes encoding the β-tubulin averting the effect of benzimidazole anthelmintics (Kotze and Prichard, 2016). As a

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change in target structure does not fully explain the whole range of resistance, other non-target site mechanisms must be involved, with non-target-site resistance (NTSR) based mainly on increased drug absorption, biotransformation and elimination. For this reason, the effect of pharmacotherapy can be decreased by the enhanced inactivation and/or elimination of active drug due to the increased expression and activity of xenobiotic-metabolizing enzymes (XME). The role of XME is the protection of the organism against the toxic action of drugs or other xenobiotics in three phases. In phase I, the drug undergoes oxidation, reduction or hydrolysis to form hydrophilic groups, while during the phase II the metabolite is conjugated with water-soluble moieties such as glucose and glutathione. These reactions increase the hydrophilicity of the xenobiotic and alter its biological reactivity. The final metabolite is transported out of cells by efflux transporters participating in phase III of drug metabolism (Matouskova et al., 2016; Hartman et al., 2021).

In the *H. contortus* genome, a relatively large number of XME has been identified (Laing et al., 2013), and the metabolism of various anthelmintics has been described (Stuchlikova et al., 2014, 2018). The predominant biotransformation reaction of benzimidazole anthelmintics is glycosylation, i.e. conjugation with UDP-activated sugar donors catalysed by UDP-glycosyltransferases (UGTs) (Stuchlikova et al., 2018). The identification of glucosides as metabolites of anthelmintics has revealed UDP-glucose as the preferred sugar donor in nematodes, as opposed to UDP-glucuronic acid in vertebrates (Bock, 2003; Stuchlikova et al., 2018). Interestingly, a higher number and greater amounts of these glucosides were found in the resistant strains compared to the susceptible strain, a finding which indicates the connection between the increased glycosylation of anthelmintics and anthelmintic resistance (Vokral et al., 2012, 2013; Stuchlikova et al., 2018; Stasiuk et al., 2019). In resistant strains of *H. contortus*, increased constitutive expression of several UGT genes was found (Matouskova et al., 2018; Kellerova et al., 2020a). In the free-living nematode *Caenorhabditis elegans,* the nonsense point mutation of *ugt-22*, which catalyses deactivation of anthelmintic albendazole (ABZ), increased the efficacy of ABZ (Fontaine and Choe, 2018). In addition, the contact of the *H. contortus* adults with sub-lethal doses of ABZ (1 μM and lower) leads to the induction of several UGTs, and subsequently to improved ABZ deactivation due to a higher production of inactive glucosides (Kellerova et al., 2020b; Dimunová et al., 2022). Phenobarbital (PHB), a classical inducer of UGTs and other XMEs, increased metabolism of anthelmintic naphthalophos in *H. contortus* larvae (Kotze et al., 2014); nothing is known, however, about the effect of PHB on ABZ metabolism in *H. contortus*.

Similarly, no information is available about the effect of UGT inhibitors on ABZ metabolism in this parasite, although metabolic inhibitors might reduce the anthelmintic biotransformation and improve drug activity. For example, co-incubation of nematodes larvae with the anthelmintic drug triclabendazole and metabolic inhibitors (piperonylbutoxide and verapamil) made larvae more susceptible to treatment (AlGusbi et al., 2014). In *H. contortus* larvae, UGT inhibitors potentiated the effect of naphthalophos (Kotze et al., 2014). The potential of UGTs inhibition as a treatment was also explored in the parasitic nematode *Brugia malayi* (Flynn et al., 2019). The specific inhibitors of mammalian UDP-glucuronosyltransferases, 5-nitrouracil (5-NU), 4,6-dihydroxy-5--nitropyrimidine (DNP), and sulfinpyrazone (SP) have commonly been used in many studies of UGT activity in various organisms (Kotze et al., 2014; Li et al., 2017).

The present study aimed to utilise enzyme induction and inhibition experiments in order to provide a deeper understanding of the role of UGTs in ABZ metabolism in *H. contortus* adult nematodes. The induction effect of PHB, ABZ and ABZ-sulphoxide (ABZSO, the main metabolite of ABZ) on UGT expression and UGT activity toward ABZ was studied *ex vivo* in isolated adult nematodes. The effect of the three potential UGT inhibitors 5-NU, DNP, and SP on ABZ glycosylation was tested *in vitro* (in subcellular fractions from nematode homogenate) and the effect of SP also *ex vivo*.

2. Materials and methods

2.1. Chemicals

Albendazole (ABZ), albendazole sulfoxide (ABZSO), mebendazole (MBZ), phenobarbital (PHB) and the tested inhibitors 5-nitrouracil (5-NU) 4,6-dihydroxy-5-nitropyrimidine (DNP), and sulfinpyrazone (SP) were purchased from Sigma-Aldrich (Prague, Czech Republic). Acetonitrile (ACN) at UHPLC-MS quality was purchased from VWR International s.r.o. (Prague, Czech Republic). Dimethyl sulfoxide (DMSO) (ACS reagent, ≥99.9%) formic acid (FA) (LC-MS LiChropur™, 97.5–98.5%) were purchased by Sigma-Aldrich (Prague, Czech Republic). Ultra-pure water was prepared from deionized water using the Milli-Q Ultrapure Water System (Millipore, Bedford, MA, USA).

Liquid sterile-filtered medium RPMI-1640 was obtained from Biosera (Biotech, Prague, Czech Republic).

2.2. Parasites

In this study, the inbred susceptible-Edinburgh strain (MHco3) ISE of *H. contortus* was used (Roos et al., 2004). Adult nematodes were isolated as before (Kellerova et al., 2020b) in agreement with Czech slaughtering rules for farm animals and according to protocols evaluated and approved by the *Ethics Committee of the Ministry of Education, Youth and Sports* (Protocol MSMT-11260/2019-2).

2.3. The exposure of adults to ABZ, ABZSO or PHB

Adult nematodes, 15 males and 10 females, were each placed into one well in 24-well plates (TPP® tissue culture plates, Sigma-Aldrich) with 2 mL of RPMI 1640 medium containing 0.8% glucose, 0.25 μg/mL amphotericin B, 10 U/mL penicillin, 10 μg/mL streptomycin, 10 mM HEPES, prepared according to a previous study (Kotze and McClure, 2001) and maintained at 37 °C with 5% CO_2 in an incubator (D180-P CO_2 Incubator, RWD Life Science Inc., USA). All the samples were prepared in 4 biological replicates.

For transcriptional analysis, the nematodes were incubated in the medium with 10 μM ABZ or 10 μM ABZSO or 2 mM PHB pre-dissolved in dimethyl sulfoxide (DMSO), with the final concentration of DMSO in the medium 0.1%. Nematodes incubated in a medium with 0.1% DMSO was used as control. After 4-h and 12-h exposure, the nematodes were immediately placed into 1 mL TriReagent® (Molecular Research Centre, OH, USA) and stored at −80 °C.

For analysis of *in vitro* ABZ metabolism, approximately 1 g of the adult nematodes (mixed females and males) were stored at −80 °C for further preparation of subcellular fraction.

For analysis of *ex vivo* ABZ metabolism, two groups of males and females (induction with ABZ or PHB) were prepared. To induce ABZ metabolism, 1 μM ABZ or 2 mM PHB was used as pre-incubation for 18 h and 4 h, respectively. For each group, a pre-incubation control was performed with 0.1% DMSO (non-induced samples). After pre-incubations, the nematodes were manually washed in PBS and placed in fresh medium with 10 μM ABZ or 10 μM ABZ and 1 mM inhibitor SP. The concentration of ABZ non-lethal to nematodes was chosen based on metabolism studies (Vokral et al., 2013; Stuchlikova et al., 2018), and the concentration of 1 mM of inhibitors was used in a previous study (Kotze et al., 2014). The incubation lasted 12 h for the ABZ induced samples and 20 h for the PHB induced samples (**Table 1**). After incubation, the medium and the nematodes washed in PBS were separately transferred into plastic tubes and stored at −20 °C. Chemical blanks (medium with anthelmintic, without nematodes) as well as biological blank samples (medium with nematodes, without anthelmintics) were prepared in the same way.

Table 1
Experimental design for the functional analysis.

	Inhibition after induction with sub-lethal dose of ABZ		
	Control	Induced	Inhibited
Pre-incubation **18 h**	0.1% DMSO	**1 μM ABZ**	1 μM ABZ
	Washing		
Incubation **12 h**	10 μM ABZ	10 μM ABZ	10 μM ABZ + **1 mM SP**
	Inhibition after induction with PHB		
	Control	Induced	Inhibited
Pre-incubation **4 h**	0.1% DMSO	**2 mM PHB**	2 mM PHB
	Washing		
Incubation **20 h**	10 μM ABZ	10 μM ABZ	10 μM ABZ + **1 mM SP**

2.4. Transcriptional analysis

RNA extraction, cDNA synthesis and quantitative qPCR was performed according to previous studies (Kellerova et al., 2020a, 2020b). In brief, the samples were homogenized in the FastPrep-24 5G Homogenizer (MP Biomedicals, France); using TriReagent® the total RNA was extracted according to the manufacturer's protocol. Half μg of the total RNA treated with DNase I (NEB, UK) was used to follow reverse transcription realized by random hexamers and Protoscript ® II Reverse Transcriptase (NEB, UK) according to the manufacturer's protocol. The obtained cDNA was diluted 10 × to a final concentration of 12.5 ng/μL and stored at −20 °C.

qPCR was performed in the 384-Well PCR Thermal Cycler; QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems, CA, USA) with SYBR Green I detection as described previously (Kellerova et al., 2020b). Two housekeeping genes, glyceraldehyde-3P-dehydrogenase (gpd) and nuclear-cap binding protein subunit 2-like (ncbp), were used as reference genes, with 31 UGT primer sets used based on a previous study (Kellerova et al., 2020a).

2.5. Preparation of subcellular fractions

One gram of the adult nematodes was mixed with 0.1 M Na-phosphate buffer, pH 7.4, and homogenized with a Potter-Elvehjem Tissue homogenizer. The subcellular fractions were obtained by differential centrifugation. The mitochondrial-like fraction was solubilized from the pellet after centrifugation at 20,000×*g*, 60 min, 4 °C in 1 mL 0.1 M Na-phosphate buffer with 20% glycerol. The supernatant from the previous step was ultra-centrifugated at 105,000×*g*, 65 min, 4 °C and the pellet of the microsomal-like fraction was resuspended by sonication in 1 mL 0.1 M Na-phosphate buffer with 20% glycerol. The prepared samples were stored at −80 °C. Before further analysis, the protein concentration was measured using a bicinchonic acid assay (BCA, Sigma-Aldrich, Prague, Czech Republic) according to the manufacturer's protocol.

2.6. ABZ glycosylation in subcellular fractions

The microsome-like (MIC) and mitochondria-like (MIT) fractions were used for the glycosylation assay, which was performed in 100 mM Na-phosphate buffer, pH 7.4 supplemented with 5 mM MgCl2, according to the modified method in previous studies (Letelier et al., 2005; Walsky et al., 2012). The composition of the enzymatic reaction is described in Table 2. To active MIC or MIT, the detergent Slovasol (Slovchema, Prievidza, Slovak Republic) was added in ration 1:2 (μg detergent: μg protein) to improve the access substrate to the membrane-bound enzyme, with the samples remaining at 4 °C for 20 min. The reactions contained 0.4 mg/mL of enzyme source (total protein content). ABZ and inhibitors were added as a solution in DMSO (total contain 2% DMSO per reaction), with the mixtures prewarmed for 5 min at 37 °C thermostated heat block before reaction initiation by adding 2 mM UDP-glucose. The samples were incubated 20 h at 37 °C, with the length of time chosen based on the production of a sufficient amount of glycosides. The three types of control – without substrate, UDP-glucose or MIC/MIT – were performed simultaneously. The reactions were stopped by adding 100 μL iced-cold acetonitrile, with the reaction tubes kept at −20 °C for 30–60 min, following which the internal standard (IS) MBZ (5 μM final concentration) was added and the samples centrifuged at 16,000×*g*. The supernatant was filtrated with a disposable syringe with a 0.22 μm filter and placed into glass vials for UHPLC-MS/MS analysis.

2.7. Analysis of ABZ metabolism

2.7.1. Sample preparation

For the nematode samples, the solid phase extraction procedure (SPE) was used in the clean-up step to obtain cleaner extracts. The Visiprep SPE vacuum manifold (Supelco®, 12-ports, PA, USA) along with SPE Strata-X, 60 mg/mL columns (Phenomenex, Torrance, CA, USA) were utilized. Before the SPE extraction, the nematode samples were homogenized using a beads homogenizer (FastPrep 24, MGP, Santa Ana, USA).

The nematode samples were resuspended in 1200 μL of Na-buffer containing 0.1 μM IS (MBZ) and homogenized using 1.4-1.0 ceramic beads, with six 30-sec cycles at 6 m/s rpm applied. The homogenized samples were centrifuged at 3000×*g* for 5 min. For protein quantification and correction, 50 μL of supernatant was kept in micro tubes and stored at −20 °C for the further measurement of the protein concentration using a BCA assay according to the manufacturer's protocol.

SPE columns were conditioned according to manufacturer's protocol by 1 mL ACN, equilibrated with 1 mL dH_20. In the next step, 1 mL of nematode homogenate or medium with IS (0.1 μM) was loaded onto the column. In an additional step, the column was washed with 2 mL 10% ACN and dried under a vacuum, after which the analytes were eluted with 1 mL ACN. The eluates were then placed in glass vials and the solvent evaporated to dryness in a vacuum rotary evaporator at 30 °C (Eppenodorf concentrator plus, Hamburg, Germany).

A reconstitution procedure was applied before the UHPLC analysis. The dry supernatant residue was reconstituted in 30 μL of ACN, followed by 5 min on a tube roller (Mx-T6-S, Dlab, CA, USA), followed by a sonication bath for 5 min. Afterwards, 70 μL of dH_20 was added followed using the same steps. The reconstituted samples (100 μL in total) were filtered through PTFE syringe filters (mesh size 0. 22 μm) and put into a glass insert.

2.7.2. LC–MS/MS conditions

The UHPLC-MS/MS analysis was conducted on the Nexera coupled LC-MS-8030 triple quadrupole analyzer (Shimadzu, Kyoto, Japan), with the column temperature maintained at 40 °C. The column employed for all analyses was the Zorbax RRHD plus C18 150 × 2.1 mm, 1.8 μm particle size with a guard column (Agilent Technologies, Waldbronn, Germany). The injection volume for all analyses was 1 μL. The samples were separated by a linear gradient elution as described before (Kellerova et al., 2020b). The mass spectrometer was operated in positive SRM acquisition mode. The settings for Shimadzu LC-MS 8030 were set as follows: spray voltage 4.5 kV, heat block temperature 400 °C, DL line

Table 2
The composition of the reaction mixture for studying ABZ glycosylation *in vitro*.

	ABZ (μM)	Inhibitor (μM)	DMSO (%)	MIC/MIT (mg/mL)	Na-phosphate buffer, pH7.4 (mM)	$MgCl_2$ (mM)	UDP-glucose (mM)
Final concentration	25	25 and 50	2	0.4	100	5	2

250 °C, flow rates of nebulizing and drying gas 3.5 L/min and 12 L/min, respectively. Nitrogen was used as the nebulizing gas and drying gas. Argon was used as a collision gas for the MS/MS experiments. As the analytical standards of the ABZ glycosides are not commercially available, semi-quantification was applied as the method of choice, with the ABZ metabolites semi-quantified using a ratio of the peak area of the metabolite with the peak area of the IS (MBZ). This ratio was normalized to 1 mg of total protein (homogenates) or 1 mL of cultivation media. Data acquisition and data processing were conducted by using LabSolution LCMS software ver. 5.109 (Shimadzu, Kyoto, Japan). Biological and chemical blanks were prepared from the medium as well as the nematode matrices.

2.8. Statistical analysis

Statistical comparisons were carried out using GraphPad Prism® software 9.3.1 (GraphPad Prism, USA). The differences between the induced samples and respective controls (normalized value = 1) represented by mean ± S.D. (with four biological replicates) in the transcriptional analysis were analysed with the Student's t-test and considered significant at $P < 0.05$. Similarly, the *ex vivo* analysis was statistically evaluated by the Student's t-test and considered significant at $P < 0.05$. The *in vitro* analysis data were analysed by one-way ANOVA with a *post hoc* Holm-Sidak's multiple comparison test; data represent the mean ± S.D. (n = 4), with differences considered significant at $P < 0.05$.

3. Results

3.1. Transcriptional response of UGTs to ABZ, ABZSO and PHB exposure

The expression levels of UGT genes in adults of *H. contortus* exposed to 10 μM ABZ, the standard dose used for metabolism analysis, in comparison with control nematodes incubated with DMSO were analysed (Fig. 1). An increased mRNA level of the gene UGT368A1 was detected in the females and no change in the males after 4 h incubation. After 12 h incubation, only UGT365B3 and UGT367A1 were induced in the females and males, respectively.

The exposure of adults with 10 μM ABZSO, the main active metabolite of ABZ, caused the induction of UGT10B1 in the females after both exposure times; in addition, UGT368B2 was induced after 12h. In the males, UGT367A1 was induced after 4 h, and three other UGT genes (UGT26A2, UGT365A1, UGT365B1) after 12h.

The effect of 2 mM PHB on the expression of UGT genes was also observed after 4 and 12 h incubation. Interestingly, the expression of UGT367A1 induced by ABZSO and ABZ in the males, was elevated after 4 h exposure with PHB in the females and males. Similarly, 12 h incubation caused the increased expression of UGT366C1 in both sexes. Besides these UGTs, UGT365A1, previously induced by ABZSO and ABZ in different sexes, was upregulated after 4 h exposure with PHB and UGT10B1 after 12 h incubation in the females. In the males, UGT365B1 was down regulated in response to 12 h incubation with PHB.

3.2. The analysis of ABZ glycosylation in vitro

In subcellular fractions of adults (MIC and MIT), the ability to form glycosides of ABZ with UDP-glucose was analysed using UHPLC-MS/MS. The N-glycosides of ABZ were identified based on their fragmentation ions as described previously (Stuchlikova et al., 2018); in addition, the analogously named M7, M8, M9 **(Table 3)** were also detected in both fractions **(Supplementary 1)**.

Initially, the effect of the three UGT inhibitors (5-NU, DNP, SP) in two concentrations 25 and 50 μM on UGT activity was tested. Decreased formation of ABZ glycosides was observed only in response to SP in both fractions **(Supplementary 2)**. Subsequently, the effect of SP in a wider concentration range (10–100 μM) on UGT activity was analysed. Concentration-dependent decrease of production of all glycosides was observed (Fig. 2) in MIC as well as in MIT.

3.3. The induction and inhibition of UGTs in adults ex vivo

To study the induction effect of ABZ and PHB on the UGT activity, ABZ-glycosides formed by *H. contortus* adults *ex vivo* were analysed separately in nematode homogenates and in medium samples. The induction experiment was performed by the pre-incubation of nematodes with 1 μM ABZ for 18 h (as previously used in (Kellerova et al., 2020b) or 2 mM PHB for 4 h and subsequent incubation with 10 μM ABZ. We have focused on the effect of pre-incubation on the formation of N-glycosides (designated as M7, M8, M9) created by direct conjugation of ABZ with UDP-glucose (Fig. 3). In homogenates of the females pre-incubated with ABZ, a higher amount of M7 was detected in comparison to non-induced controls. In the medium of females pre-incubated with ABZ, a higher

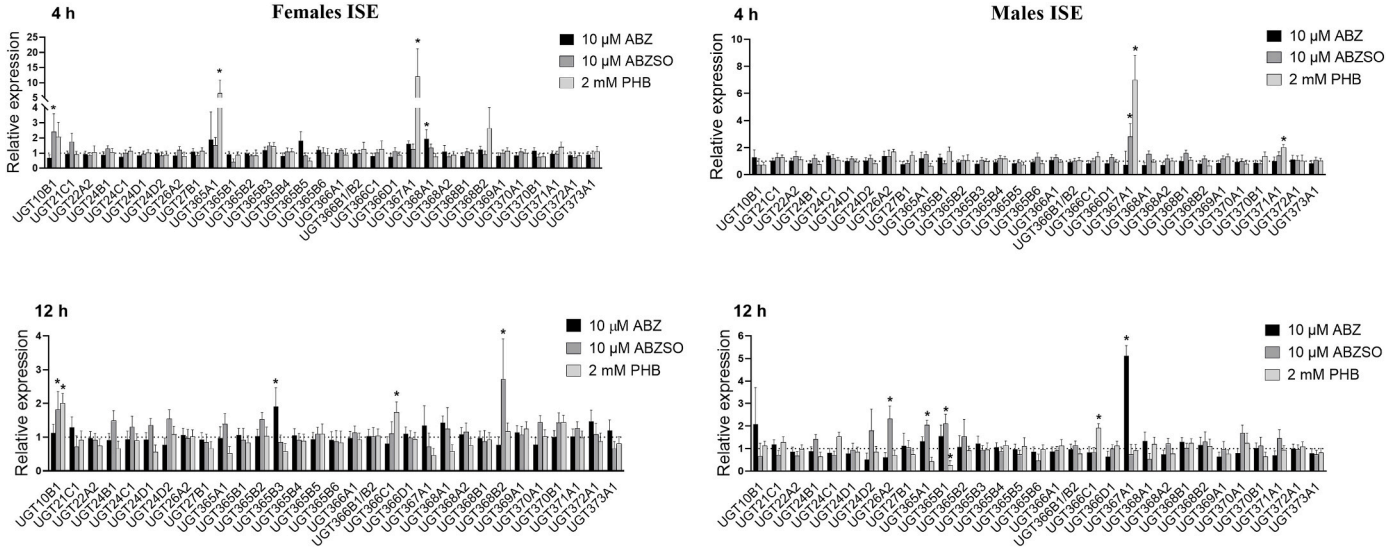
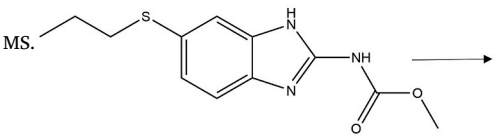


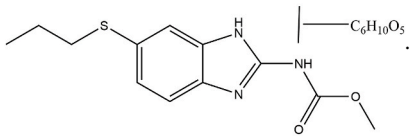
Fig. 1. Expression levels of UDP-glycosyltransferases (UGTs) mRNA in female and male *H. contortus* adults ISE strain in response to 10 μM ABZ, 10 μM ABZSO and 2 mM PHB for 4 and 12 h. The reference genes for gene expression normalization were glyceraldehyde-3P-dehydrogenase (gpd) and the nuclear-cap binding protein subunit 2-like (ncbp), control (=1) is represented by a dotted line. Data represent mean ± S.D. (n = 4), *$P < 0.05$.

Table 3
Biotransformation of ABZ in *H. contortus* adults – the glycosides M7, M8, M9 detected by UHPLC-MS/MS.

ABZ
$C_{12}H_{15}N_3O_2S$



M7 – M9
$C_{18}H_{25}N_3O_7S$



t_R (min)	Theoretical *m/z* values of [M+H] + ions	Elemental composition	Description of metabolite formation	Product ions of [M+H] + *m/z*, (collision energy)	Metabolite designation
5.1	428.15	$C_{18}H_{25}N_3O_7S$	N - glycosylation	266.1 (−21), 234.1 (−37)	M7
5.6	428.15	$C_{18}H_{25}N_3O_7S$	N - glycosylation	266.1 (−21), 234.1 (−37)	^{ABZ}M8
6.1	428.15	$C_{18}H_{25}N_3O_7S$	N - glycosylation	266.1 (−21), 234.1 (−37)	^{ABZ}M9
6.8	266.10	$C_{12}H_{15}N_3O_2S$	–	234.00 (−21), 190.85 (−33) 158.90 (−39)	^{ABZ}ABZ
6.9	296.00	$C_{16}H_{13}N_3O_3$	–	263.85 (−23), 105.05 (−36) 77.00 (−49)	MBZ

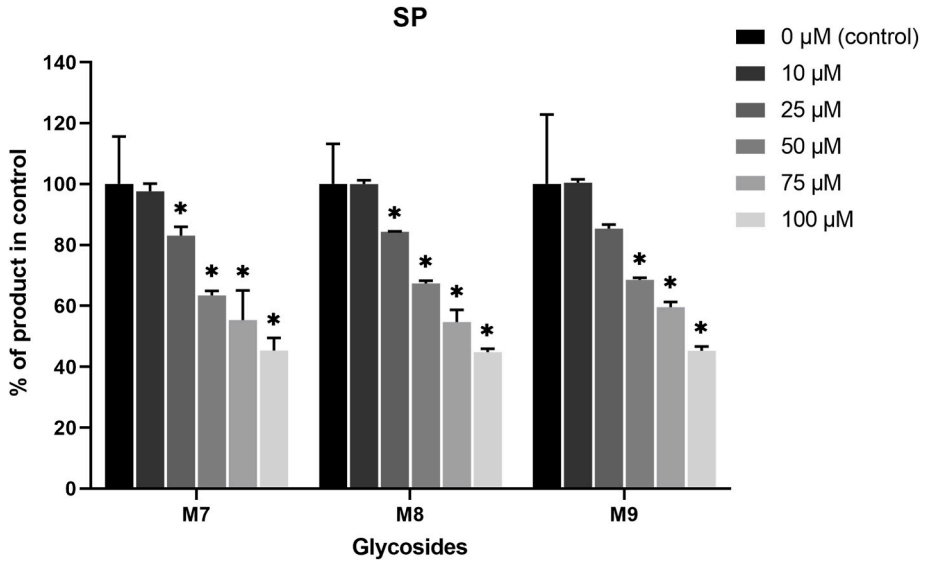


Fig. 2. The production of N-glycosides (M7, M8, M9) inhibited by suplfinpyrazone (SP) in a microsome-like fraction related to the control reaction without an inhibitor that represents 100% of the formed product. The data were analysed by one-way ANOVA and represent the mean ± S.D. (n = 4), *P < 0.05.

amount of M7 and M8 were found, which indicates enhanced excretion of M8 from the nematodes to the medium. Similarly, in the homogenate of ABZ-induced males, the amount of M8 increased in comparison to controls, while in the medium of ABZ-induced males a higher amount of all ABZ metabolites was detected. ABZ pre-incubation also induced ABZ oxidation, i.e. formation of ABZSO and ABZ sulfone (M3 and M6, **Supplementary 3,4**). On the other hand, no induction effect of PHB on ABZ metabolism was observed.

To confirm the inhibitory effect of SP *ex vivo*, the nematodes (pre-incubated with ABZ or PHB) were incubated with 1 mM SP. The inhibition effect on the formation of all analysed glycosides was observed both in the nematode homogenates and in the media of both sexes, proving SP as a potent inhibitor of UGTs in *H. contortus* (Fig. 4).

4. Discussion

Glycosylation represents the predominant conjugation reaction which organisms, especially nematodes, use in the detoxification of foreign compounds (Stasiuk et al., 2019). UDP-glycosyltransferases (UGTs) represent a type of biotransformation enzyme that catalyze the transfer of glycosyl residues from activated sugar donors to acceptor molecules. The basic information about the superfamily of UGTs in *C. elegans* and *H. contortus* has been summarized (Matouskova et al., 2016; Hartman et al., 2021), but a closer look at the UGTs in nematodes has been undertaken only rarely, although the formation of glycosylated metabolites of several anthelmintics has been reported (Cvilink et al., 2008; Vokral et al., 2012, 2013). The importance of UGTs in ABZ efficacy in *C. elegans* was identified for the detoxification gene *ugt-22* (UGT16C1, clusters with Hco_UGT365 isoforms), which belongs to a group of rapidly evolving and expanding UGT subfamily. In the presence of ABZ, the nonsense mutation of *ugt*-22, was found to cause reduced worm motility; *vice-versa*, the overexpression experiment showed increased worm motility, *i.e.* enhanced survival (Fontaine and Choe, 2018). The transcription level of UGTs in *H. contortus* fluctuates during ontogenetic development, with most of the UGTs showing a higher expression in juvenile stages, possibly due to the greater levels of environmental toxins to which the free-living stages are exposed (Kellerova et al., 2020a). The connection between UGTs and anthelmintics resistance in *H. contortus* has been suggested due to the enhanced production of glycosides (Stuchlikova et al., 2018) and higher constitutive expression of UGT368B2 in resistant than in sensitive strains (Matouskova et al., 2018).

In the present study, we analysed the expression of UGT genes in response to ABZ (10 µM) and ABZSO (10 µM). As generally

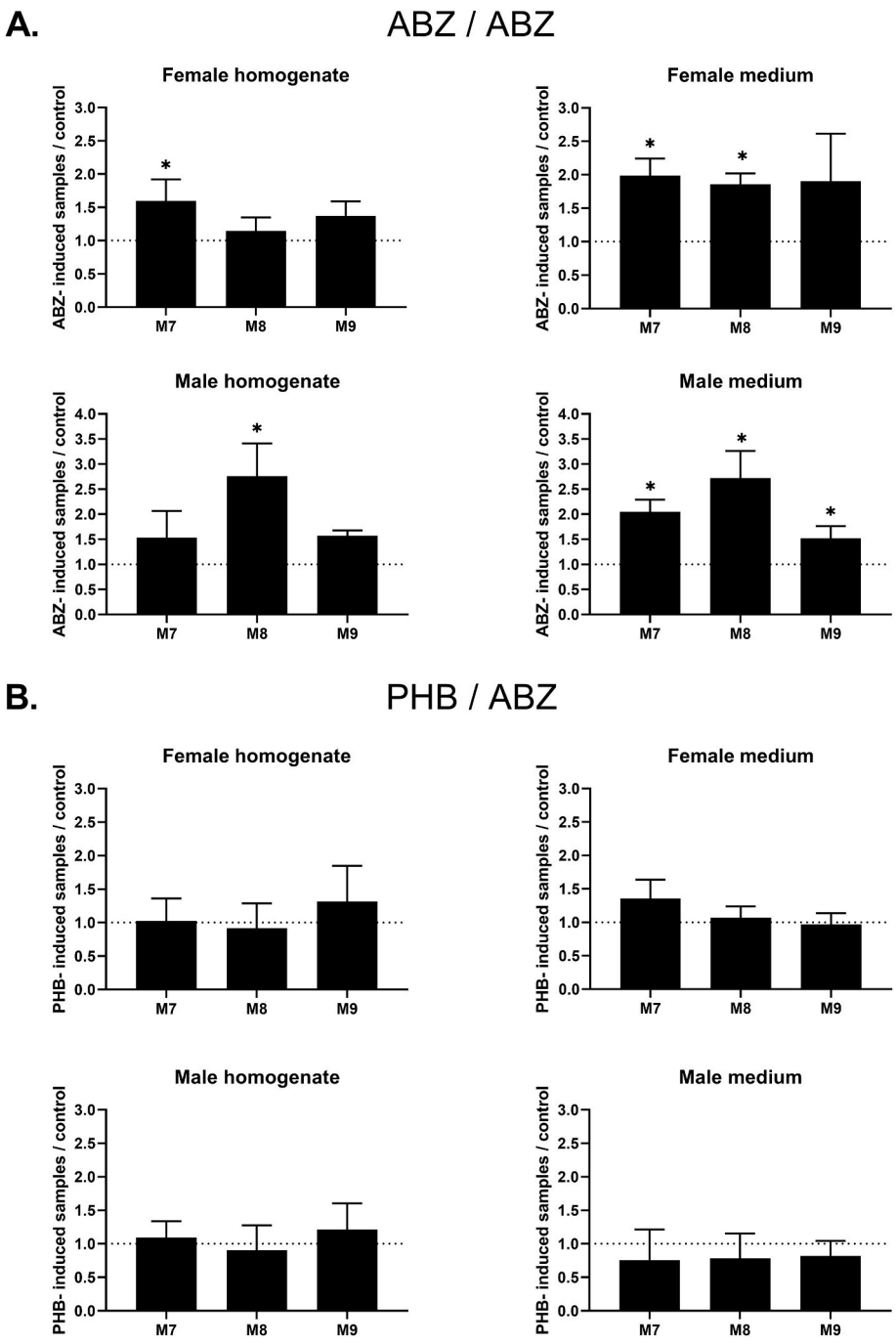


Fig. 3. The induction effect of 1 μM albendazole (ABZ) and 2 mM phenobarbital (PHB) on the production of ABZ glycosides. The comparison of the amounts of glycosides M7, M8, M9 presented in the homogenate and cultivation medium of females and males of *H. contortus*: **A.** after 12 h incubation in 10 μM ABZ with prior 18 h pre-incubation with 1 μM ABZ related to control with DMSO pre-incubation instead of a low dose of ABZ, **B.** after 20 h incubation in 10 μM ABZ with prior 4 h pre-incubation with 2 mM PHB related to control with DMSO pre-incubation instead of PHB. The data represent the mean ± S.D. (n = 4). An asterisk (*) indicates a significant difference ($P < 0.05$) compared to control (with value one displayed as a dotted line in graphs).

recommended for a transcriptional response study, two exposure times (4 h and 12 h) were used. Not many UGTs were upregulated by ABZ nor ABZSO after 4-h exposure both in the males and females, with more transcripts upregulated in males after 12 h. Similarly, the 4-h exposure to ABZ did not lead to any induction of UGTs in the juvenile stages (Kellerova et al., 2020a), a result also found with ABZ in very high concentrations in *H. contortus* adults (Stasiuk et al., 2019). On the other hand, the exposure of *H. contortus* adults to sub-lethal concentrations of ABZ and ABZSO (1 μM and lower) for 4 and 12 h *ex vivo* (Kellerova et al., 2020b), and for several weeks *in vivo* (Dimunová et al., 2022) led to the transcriptional response of several UGTs (Table 4). The upregulation of UGT368A1 after 4-h exposure to 10 μM ABZ observed in present study in *H. contortus* adults has also previously been found in *H. contortus* larvae L3 (Kellerova et al., 2020a). In adult females, ABZSO at 10 μM induced UGT10B1, as was also the case at all sub-lethal concentrations (Kellerova et al., 2020b). Similarly, ABZSO either at 10 μM or at sub-lethal doses induced UGT26A2 in males (Kellerova et al., 2020b). Interestingly, in males, UGT367A1 was upregulated by ABZ only after 12-h exposure, while ABZSO induced the expression faster. However, in nematodes exposed to ABZ, the actual effect of ABZSO cannot be ruled out, since it is the main metabolite of ABZ and may remain present in a sufficient amount after 12-h exposure. UGT368B2, which has shown a higher constitutive expression in adults of resistant strain (Matouskova et al., 2018), was induced in females exposed to 10 μM ABZSO. The induction of this gene by sub-lethal doses of ABZ in females and males has been observed previously (Kellerova et al., 2020b). The induction of UGTs genes by ABZ (1 μM) resulted in enhanced formation of ABZ-glycosides as well as their efflux from the nematodes' bodies. As

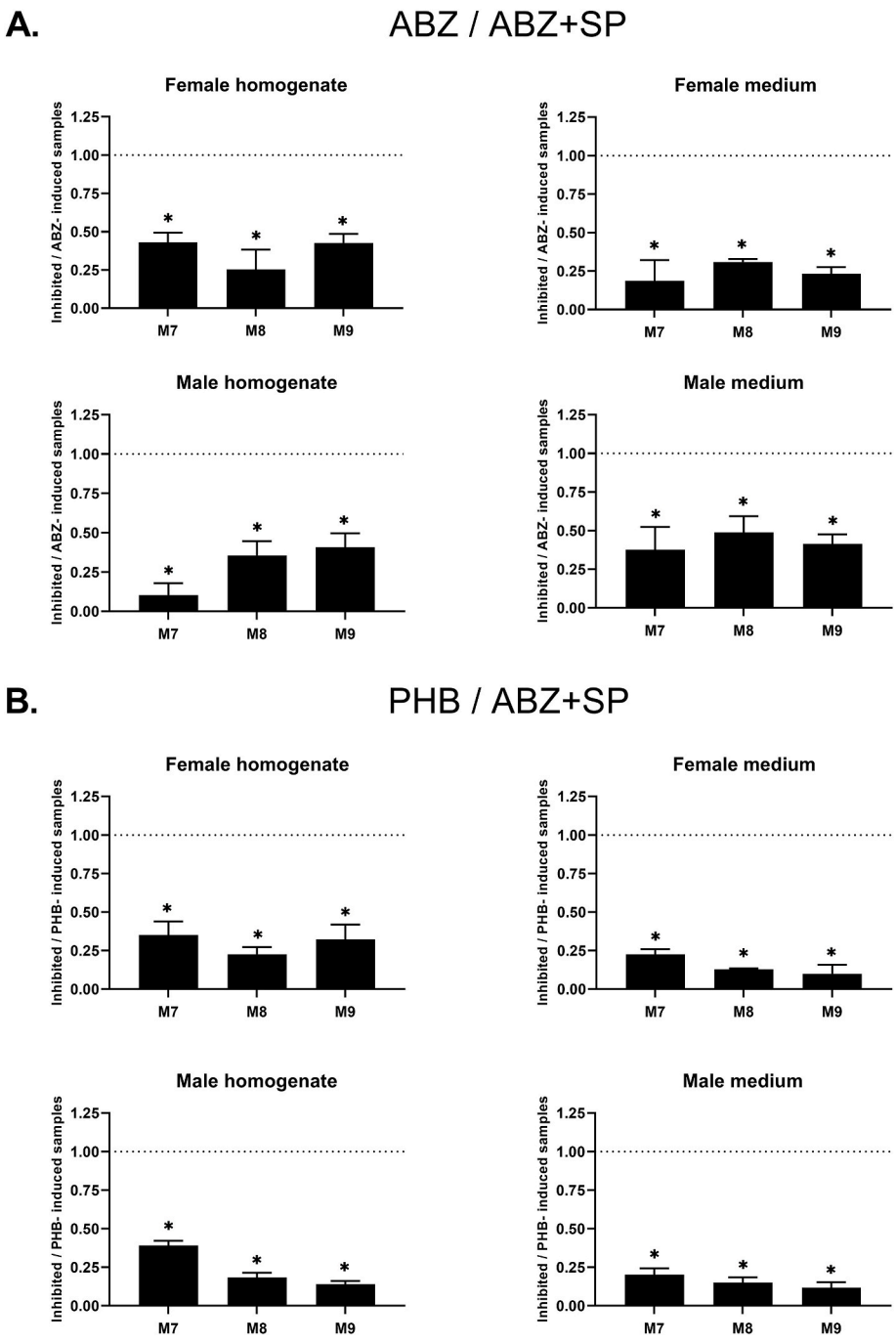


Fig. 4. The inhibition effect of 1 mM sulfinpyrazone (SP) on the production of ABZ glycosides. The comparison of the amounts of glycosides M7, M8, M9 presented in homogenate and cultivation medium of females and males of *H. contortus*: **A.** after 12 h incubation in 10 μM ABZ and 1 mM SP with prior 18 h pre-incubation with 1 μM ABZ related to control without SP, **B.** after 20 h incubation in 10 μM ABZ and 1 mM SP with prior 4 h pre-incubation with 2 mM PHB related to control without SP. The data represent the mean ± S.D. (n = 4). An asterisk (*) indicates a significant difference ($P < 0.05$) compared to control (with value one displayed as a dotted line in graphs).

can be seen from the summary in Table 4, sub-lethal concentrations either *in vivo* or *ex vivo* lead to induction of more UGTs in general. The concentration of ABZ/ABZSO (10 μM) used in this study, what is commonly used for detection of metabolites, increased only few transcripts in *H. contortus* adults. Possible reason for such discrepancy can be that other genes (e.g. genes responsible for oxidation-reduction processes) are initially increased to cope with this concentration of ABZ to avoid its detrimental effect. Similarly, the induction study by Stasiuk et al. (2019) that used even higher concentrations of benzimidazoles (1.13 mM) did not induce many transcripts as well. True is that the adult parasites *ex vivo* treatment is far from the normal environment in sheep abomasum and the feeding can be disrupted, on the other hand, the sub-lethal treatments were performed under the same conditions.

We also exposed females and males of *H. contortus* to PHB to study the effect of this classical inducer of XMEs on UGTs expression and ABZ metabolism in nematodes. We chose the PHB concentration (2 mM) based on the PHB-induced drug tolerance experiments, where the role of UGTs was indirectly confirmed in *H. contortus* larvae by synergism effect with inhibitors and anthelmintic naphthalophos (Kotze et al., 2014). However, the effect of PHB hasn't been tested on adult nematodes before. In our experiments, only a few UGTs genes were induced by PHB, e.g. UGT365A1 in the females and UGT367A1 in both sexes after 4h. As previously reported ABZ in sub-lethal concentrations tested in the same conditions (i.e., *ex vivo*, 4 and 12 h of exposure) induced more UGTs than PHB (Kellerova et al., 2020b). However, UGT365A1 and UGT367A1 we also induced suggesting that PHB and ABZ control the expression of these UGTs through a similar mechanism, although nothing is known about the regulatory areas of the affected UGTs nor the presence of

Table 4
Summary of induced UGTs in *H. contortus* adults after exposure to ABZ, ABZSO and PHB. UGTs induced more than two fold are displayed in bold.

Treatment	Females	Males	References
sub-lethal ABZ (4 h/ 12h)	**UGT365A1** **UGT367A1** UGT368B2 **UGT10B1**	UGT26A2 UGT365A1 UGT365B1 UGT365B3 UGT365B5 UGT366C1 UGT368A2 **UGT368B2** **UGT371A1**	Kellerova et al. (2020b)
sub-lethal ABZSO (4 h/ 12h)	**UGT10B1** UGT24D1 UGT24D2 UGT366A1 UGT366C1 UGT372A1	**UGT24C1** UGT26A2 UGT365B5 UGT366C1 UGT368B1 UGT369A1 UGT371A1 **UGT372A1**	Kellerova et al. (2020b)
ABZ *in vivo*	**UGT365B1** UGT365B5 UGT366B1/ B2 UGT368B2	**UGT24B1** **UGT24C1** UGT26A2 **UGT365B1** UGT365B5 UGT368A1 **UGT368A2** UGT368B2 UGT370B1	Dimunová et al. (2022)
10 µM ABZ (4 h/12h)	UGT368A1 **UGT365B3**	**UGT367A1**	[a]
10 µM ABZSO (4 h/12h)	UGT10B1 **UGT368B2**	UGT26A2 UGT365A1 UGT367A1 UGT365B1	[a]
2 mM PHB (4 h/12h)	UGT10B1 **UGT365A1** UGT366C1 **UGT367A1**	UGT366C1 UGT367A1 UGT371A1	[a]

[a] included in this study.

xenobiotic responsive elements in the *H. contortus* genome. The PHB-mediated induction of UGTs did not result in the increased formation of ABZ-glucosides. Possibly other UGTs than those induced by PHB are responsible for ABZ metabolism.

In the second part of our study, the possible inhibition of UGT activity toward ABZ in *H. contortus* was tested using common UGT inhibitors. The application of specific inhibitors is a common strategy to verify a function of a specific enzyme in a certain reaction. For example, in triclabendazole resistant *Fasciola hepatica* the roles of flavin monooxygenases and cytochromes P450 were proved using the inhibitors methimazole (Devine et al., 2009) and piperonyl butoxide (Devine et al., 2011), respectively. Verapamil, a well-known inhibitor of efflux transporters P-glycoproteins (Pgp), reversed the resistance to ivermectin in *Fasciola hepatica* (Savage et al., 2013) and *H. contortus* (Borges et al., 2011). The UGT inhibitors 5-NU, DNP and SP have often been used in various organisms to demonstrate the role of UGTs in resistance. In the insect *Aphis gossypii*, SP and 5-NU significantly increased the toxicity of thiamethoxam in the resistant strain (Pan et al., 2018); in *Diaphorina citri*, exposure to these UGT inhibitors increased the toxicity of imidacloprid in the resistant population (Tian et al., 2019), and a similar analysis was performed in chlorantraniliprole resistant *Plutella xylostella* (Li et al., 2017). In addition to investigations with insects, the adult nematode *Brugia malay* was incubated with the SP/ABZ combination, with a significant decrease in worm motility observed, confirming the prolonged effect of ABZ (Flynn et al., 2019). Similarly, in *H. contortus* larvae, naphthalophos in combination with UGT inhibitors decreased their survival, possibly because the inhibition of biotransformation enzymes prolonged biological activity of naphthalophos (Kotze et al., 2014). To prove the involvement of UGTs in ABZ metabolism in *H. contortus* adults, we used subcellular fractions to test the effects of three inhibitors (SP, 5-NU, DNP) on ABZ metabolism. The determination of UGT activity in subcellular fraction, however, is complicated due to the not-fully-active transporters of UDP-glucose, which lead to the UGT reduced activity, a condition referred to as UGT latency *in vitro* (Fisher et al., 2000; Walsky et al., 2012); thus this analysis provides only a distorted view of UGT activity. Nevertheless, our results show that SP is the most effective inhibitor of glycosides formation in comparison with the others tested. Our examination of the effect of SP on the metabolism of ABZ tested *ex vivo* in live *H. contortus* adults also highlighted the ability of SP to reduce ABZ biotransformation. However, the specific UGT responsible for particular ABZ metabolites needs to be identified, with several interesting candidates already shown to be worth further investigation.

In conclusion, the obtained results confirmed the role of UGTs in ABZ biotransformation in *H. contortus* adults. Therefore, glucose conjugation as an important pathway of anthelmintics metabolism should be considered as a potential target for combinational therapy. Our study has revealed sulfinpyrazone as a potent inhibitor of ABZ glycosylation in this parasite, thus the possible use of sulfinpyrazone with ABZ in combination therapy deserves further research.

Authors contribution

PM designed and supervised the project; DD and PK performed the transcriptomic analysis; MA provided statistical analysis; MN and DD conducted the metabolic analysis; DD, MN and PM wrote the manuscript; LS revised manuscript.

Declaration of competing interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpddr.2022.06.001.

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