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ORIGINAL ARTICLE

Albendazole enantiomeric metabolism and binding to cytosolic proteins in the liver fluke *fasciola hepatica*

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Abstract Fascioliasis causes important economic losses in ruminant species all over the world. Its control is largely based on the use of the flukicidal compound triclabendazole (TCBZ). However, its chemically related benzimidazole anthelmintic albendazole (ABZ) is being successfully used to control TCBZ-resistance flukes. This research gains some insights into the comparative molecular behaviour of both anthelmintics within the target fluke. The goals of the current work were: (i) to assess the competitive binding of ABZ and TCBZ to cytosolic proteins of *F. hepatica,* and (ii) to evaluate the enantioselective biotransformation of ABZ in microsomal fractions obtained from TCBZ-susceptible and TCBZ-resistant strains of the liver fluke. Cytosolic proteins from fluke specimens bound TCBZ with greater affinity (83%) than ABZ (44%) and the fraction of TCBZ bound to cytosolic proteins was not displaced by ABZ. The microsomes from both -susceptible and resistant flukes sulphoxidized ABZ into ABZ sulphoxide (ABZSO). However, this oxidative activity was 49% higher in microsomes from TCBZ-resistant flukes ($P<0.001$) with a predominant production of the (+) ABZSO enantiomer. As earlier shown for TCBZ, the results reported here confirm an enhanced ability for ABZ oxidation in TCBZ-resistant flukes. While this enhanced oxidative metabolism of ABZ may cooperate to the resistance phenomenon, other pharmacodynamic-based mechanisms may be involved, which would explain why, although being chemically-related, ABZ remains efficacious against TCBZ resistant flukes under field conditions.

Keywords *Fasciola hepatica* · Albendazole · Enantiomeric metabolism · Binding to cytosolic proteins · Triclabendazole resistance

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Introduction

A wide range of modern anthelmintics, with broad-spectrum and high efficacy to control helminth infections in ruminants, are commercially available in the veterinary market. Benzimidazole (BZD) anthelmintic compounds are widely used against nematode, cestode and trematode parasites such as *Fasciola hepatica*. Fascioliasis is an important zoonotic disease, particularly in underdeveloped countries (Mas-Coma 2004). This widespread trematode disease is responsible for economic losses in ruminant species all over the world. The main strategy for the effective control of fascioliasis is still based on the use of flukicidal compounds. Most of the available flukicidal compounds, at their recommended therapeutic doses, have good activity against the mature stage of the liver fluke, but are not sufficiently effective against the pathogenic immature stages. Among the anthelmintics, triclabendazole (5–chloro–6 (2–3 dichlorophenoxy)–2–methylthio - 1H -benzimidazole) (TCBZ) is an halogenated BZD thiol derivative, which shows excellent efficacy against both juvenile (immature) and adult stages of *F. hepatica* (Boray and De Bono 1989). ABZ (methyl-[(5-propylthio)-1H-benzimidazol-2-yl] carbamate–) is also an alternative BZD compound used for the control of adult stages of the liver fluke older than 12 weeks.

Parasite resistance to different BZDs is a growing world-wide problem. The resistance of *F. hepatica* to TCBZ has now emerged in several countries. ABZ is being, to some extent, successfully used to control TCBZ-resistant flukes. This resistance to flukicides was first reported at the end of the 1980s (Boray and De Bono 1989). Since 1995, several reports of *F. hepatica* resistance to TCBZ under field conditions have been reported in Australia (Overend and Bowen 1995), Ireland (O'Brien 1998). Scotland (Mitchell et al. 1998), Wales (Thomas et al. 2000) the Netherlands (Moll et al. 2000, Gaasenbeek et al. 2001) and Spain (Alvarez-Sánchez et al. 2006). Nematode resistance to BZDs has been linked to a single amino acid substitution (phenylalanine to tyrosine) at position 200 on the beta-tubulin molecule. However, sequencing of beta-tubulin cDNAs from TCBZ -susceptible and -resistant flukes revealed no aminoacid differences between their respective primary sequences. (Robinson et al. 2002). The *Fasciola* genome encodes at least five α- and six β-tubulin isotypes that are expressed at the adult stage of the life cycle. Of the sequences identified, three α- and four β-isotypes group with the other trematode tubulins in phylogenetic analysis, whereas the others are more diverse. The determination of these sequences should allow their *in vitro* expression, which in turn may allow investigation as to which of these isotypes, if any, TCBZ binds When isotype RT-PCR fragment sequences were compared between six individual flukes from the TCBZ-susceptible Cullompton isolate and from seven individual flukes from the two TCBZ-resistant isolates, Sligo and Oberon, these residues were conserved (Ryan et al. 2008).

The BZD anthelmintics are extensively metabolized in the host (Gottschall et al. 1990). The metabolism of ABZ and TCBZ tends to convert these molecules into more polar and less active metabolites, which facilitate their elimination. This metabolic pattern and the resultant pharmacokinetic behaviour are relevant in the attainment of high and sustained concentrations of pharmacologically active drug/metabolites in the target parasite. The pharmacodynamic activity of BZD compounds depends on the sustained presence of the active drug or active metabolites at the site of the target parasite's location (Lanusse et al. 1993). Both ABZ and TCBZ are metabolized to their sulphoxide metabolites by the host liver (Virkel et al. 2004, 2006) but also by the parasite's subcellular fractions (Solana et al. 2001; Mottier et al. 2004). After ABZ and TCBZ administration to sheep, two sequential

oxidative steps in the liver generate their respective sulphoxide (ABZSO and TCBZSO) and sulphone ($ABZSO_2$ and $TCBZSO_2$) metabolites, which are the main metabolites recovered in the bloodstream. Both sulphoxides are considered anthelmintically active metabolites, while their respective sulphones are pharmacologically inactive. Likewise, sequential sulphoxidation and sulphonation were observed in subcellular fractions obtained from adult liver flukes. Moreover, it has been reported that *F. hepatica* had significantly higher sulphoxidative activity than nematode and cestode parasites (Solana et al. 2001).

The metabolite ABZSO has an asymmetric center in the sulphur atom of its molecule and two ABZSO antipodes have been detected (by chiral separation) in the plasma of sheep and cattle after administration of its parent drug ABZ (Delatour et al. 1990). Enantiomers may exhibit clear differences in their pharmaco/toxicological activity and most biochemical processes in the organism are stereospecific in nature (Landoni et al. 1997). Knowing that ABZ may be effective for the control of flukes resistant to TCBZ (Coles and Stafford 2001), the work presented here was designed to gain further insight into the comparative pharmacological behaviour of ABZ and TCBZ. Studies were devoted to characterize the competitive drug binding to cytosolic proteins of *F. hepatica* and to characterize the enantioselective metabolic fate of ABZ in TCBZ-susceptible and TCBZ–resistant strains of the liver fluke. Improving our knowledge on some biochemical pharmacology issues occurring within target parasites may contribute to explain the mechanism(s) underlying the development of resistance and to optimise the use of the BZD anthelmintics in ruminants.

Material and methods

Chemicals

Reference standards (97–99% pure) of ABZ and ABZSO were a gift from Schering-Plough, Kenilworth, NJ, USA. TCBZ was provided by Novartis Animal Health (Basel, Switzerland). Sephadex G25, PIPES (piperazine-N-N-bis-[2-ethanesulphonic acid]), Na_2HPO_4 and ammonium phosphate, the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH), tris[hydroxymethyl] aminomethane hydrochloride (Tris), were purchased from Sigma®. The solvents used for the chemical extraction and chromatographic analysis were HPLC grade (Baker Inc., Phillipsburg, USA).

Parasite species collection

Adult liver flukes were recovered from the hepatobiliary tract of untreated sheep infected 16 weeks earlier with 300 metacercariae from TCBZ-susceptible (*Cullompton*) or TCBZ-resistant (*Sligo*) strains of *F. hepatica*.. Liver fluke metacercariae were kindly provided by Professor I. Fairweather, School of Biology and Biochemistry, The Queens University of Belfast, Northern Ireland, UK. The infection was confirmed by the presence of eggs in faeces and indirect estimation of liver damage after determination of high levels of serum glutamate dehydrogenase (GLDH) and gamma glutamyl transferase (γGT) activities. Infected animals were sacrificed and adult flukes were collected from bile ducts and liver, and processed. The collection of the flukes, their processing to obtain the microsomal and cytosolic fractions and the incubation assay conditions were as described earlier (Solana et al. 2001; Mottier et al. 2004)

Preparation of microsomal and cytosolic fractions

Parasite specimens (10–15 g) of the TCBZ-susceptible or –resistant isolates of *F. hepatica* were rinsed with cold KCl (1.15%) and then transported to the laboratory in flasks filled with phosphate buffer (0.1 M, pH 7.4) at 4°C. All subsequent operations were performed between 0 and 4°C.

Microsomes (Ms) and cytosolic fractions (Cyt) of both strains of *F. hepatica* were prepared according to a technique adapted by Solana et al. (2001) from that described elsewhere for sheep, pig and cattle liver (Galtier et al. 1986; Souhaili-El Amri et al. 1987; Lanusse et al. 1993).

Each sample was cut into small pieces and washed several times with a phosphate buffer. Samples were homogenised (1:1 in phosphate buffer, pH 7.4) in an Ultra-Turrax homogenizer (IKA Works Inc., Wilmington, USA), centrifuged at 10.000 *g* for 20 minutes and the resulting supernatant centrifuged at 100.000 *g* for 60 min. The supernatant (Cyt) and the pellets (Ms), suspended in 0.01 M phosphate buffer, were collected and stored at –80°C until incubation assays analysis. Proteins content from the Ms and Cyt fractions were determined using bovine serum albumin as a standard (Lowry et al. 1951).

Metabolism assays

Sulphoxidation of ABZ was assessed by the amount of total ABZSO [(+) ABZSO plus (-) ABZSO] formed in the presence of NADPH (Sigma Chemical Co., St. Louis, MO, USA). Vials containing the Ms fractions of each parasite strain of *F. hepatica* were boiled 2 min in a water bath, and incubated simultaneosulsy with vials without the Ms preparation. The content of the reaction mixture (final volume: 1 ml) was: 1.8–2.5 μg/ml of Ms protein, 625 μM of NADPH and 15 μg of ABZ (56.55 nmol solubilized in 15 μl of methanol). The overall percentage of solvent in the incubation mixture was 1.5%. In a preliminary assay, it was observed that the plateau of the reaction was reached after 60 minutes of incubation; hence, this incubation time was chosen to evaluate parasite metabolism in the current experiments. Incubation assays were carried out in glass vials in a shaking water bath at 37°C for 60 minutes under aerobic conditions. At the end of the incubation period, the reaction was stopped by the addition of 1 ml of acetonitrile. The incubation mixtures were frozen at -20°C until processed for chemical extraction and HPLC analysis. All incubations were performed with at least five (5) replicates. The enantioselective sulphoxidation was assessed by the amount of (+) ABZSO and (-) ABZSO formed after ABZ incubation with both Ms fractions.

Drug binding to cytosolic proteins

A Sephadex G25 column was prepared according to Solana et al. (2002a). The column was hydrated with PIPES 0.1 M, pH 7.2 (1 g/10 ml) fitted in a glass column of 20×0.8 cm with a stopcock valve and glass microfiber filter. After decantation, a final volume of 8 ml was obtained from each column. The exclusion volume was determined using 1 ml of plasma as control, eluting with PIPES 0.1 M, pH 7.2. Aliquots of 0.5 ml were collected and protein content was measured at 280 nm in a Beckman spectrophotometer.

One ml of Cyt fraction obtained from flukes of the *Cullompton* strain (TCBZ-S) was incubated 60 min at 37°C in the presence of 10 μl of TCBZ (1 mg/ml methanol), 10 μl of ABZ (1 mg/ml methanol) or 10 μl of TCBZ plus 30 μl of ABZ (1 mg/ml methanol respectively). Under these conditions, some of the molecules bind to cytosolic proteins.

Each sample was immediately loaded into a Sephadex G25 column eluted with PIPES 0.1 M (pH 7.2) and 1 ml aliquots were collected. After all the protein was eluted, 1 ml fractions were collected. TCBZ and ABZ were quantified in each collected fraction.

Sample preparation, extraction and analytical procedures

The drugs (TCBZ, ABZ and ABZSO) were chemically extracted from samples using Sep Pak C18 cartridges (Water Associates, Milford, MA, USA) conditioned with 5 ml methanol (HPLC grade, Baker, NJ, USA) and 5 ml ammonium phosphate 17 mM, pH 5.5. Samples were eluted, evaporated to dryness under a nitrogen stream and analysed by HPLC (Shimadzu, Kyoto, Japan) with an UV detector (SPD-10A, Shimadzu, Kyoto, Japan) reading at 292 nm and a reverse phase C18 column (Selectosil, Phenomenex, CA, USA). The mobile phase was composed of acetonitrile/ammonium phosphate (25 mM, pH 5.5). Identification of the different molecules was done by the retention time of pure reference standards (97–99%). In drug binding assays to cytosolic proteins, the peaks of each sample were grouped depending on their elution time being collected at the beginning (bound drug) or at the end (free drug) of the Sephadex G25 elution. In the metabolic assays, the ABZSO peaks of each sample were quantified and collected for the enantiomeric study.

The same chromatographic equipment was used to quantify the ABZSO enantiomeric forms. ABZSO enantiomers were separated and identified using a chiral column. The stationary phase was an alpha-glycoprotein in silica gel (CHROMTECH, Chiral-AGP, 5 μm, 100×4.0 mm). The mobile phase consisted of 1% isopropanol, 8 mM Na2HPO4 in water. The relative proportions of the peaks, expressed as percentage, were calculated by using the integrator software.

Data analysis

The amount of drug remaining free or bound to cytosolic proteins, and the concentration of each metabolic product formed after drug incubation with the Ms fractions of the different fluke's strains were quantified using the internal standard area method. The percentage of the each enantiomeric form was calculated using the sum of the values of the amount of unmetabolized drug plus the amount of the total metabolites produced as a 100%. The data on the amount of enantiomeric products formed or bound drug after incubation of the Ms and/or Cyt of the different flukes were statistically compared using analysis of variance (ANOVA). Statistical comparisons were carried out using the Instat 3.00 software (Graph Pad Software, Inc.).

Results

The gel filtration technique using Sephadex G25 microspheres (exclusion volume 5.000 daltons) was able to separate free ABZ and/or free TCBZ from that bound to cytosolic proteins in all the assays performed in the present study. The quantification of the drugs allowed grouping the samples into two batches. One representing the lowest ABZ or TCBZ concentrations which correspond to the first eluted fractions (bound ABZ or bound TCBZ). The second batch with the highest drug concentrations being the latest fractions collected (free drug). This pattern agrees with the fact that compounds with high molecular weight (proteins with bound TCBZ or ABZ) pass through the Sephadex G25

column faster than those with low molecular weight (free TCBZ or free ABZ), which leaves the column later (Fig. 1).

Both anthelmintic molecules (ABZ and TCBZ) showed affinity for cytosolic proteins of the fluke *F. hepatica*. The fluke's cytosolic proteins bound TCBZ with greater affinity (83% of total TCBZ supplied in the medium) than ABZ (44% of the incubated drug). The fraction of ABZ bound to cytosolic proteins resulted significatively displaced by its competence with an equivalent concentration of TCBZ ($P<0.001$), whereas the fraction of TCBZ bound to cytosolic proteins was not displaced by ABZ. Displacement of TCBZ from its binding site(s) was not observed although concentrations of ABZ were 3-fold higher than the assayed TCBZ concentrations (Fig. 2, Table 1).

The microsomal fractions from both fluke strains (susceptible and resistant), oxidized ABZ into ABZSO. This oxidative activity was 49% higher in microsomes obtained from the *Sligo* strain resistant to TCBZ ($P<0.01$) (Fig. 3). A higher proportion of the (+) ABZSO enantiomer was produced by the microsomal fraction obtained from TCBZ-resistant flukes compared to that measured after ABZ incubation with microsomes from susceptible parasites (Fig. 4). The greater amount of ABZSO formed and the estimated enantiomeric excess shown in the insert of Fig. 4 are in agreement with an enhanced formation of the (+) ABZSO enantiomer in TCBZ-resistant compared to the susceptible flukes.

Discussion

Different alternatives for parasite control have been tested, including vaccines, selection of naturally resistant animals and biological control. However, chemical treatment of parasites

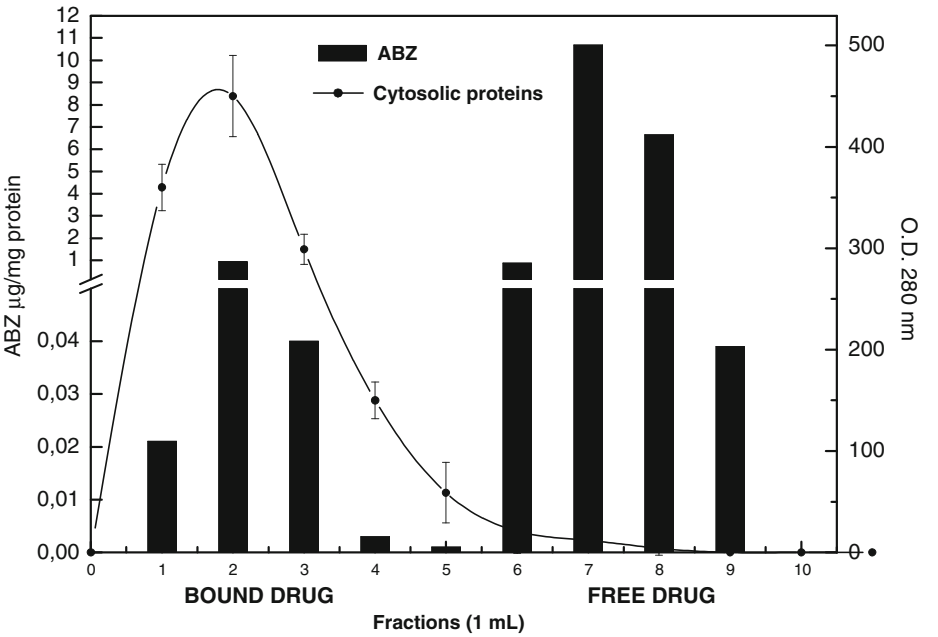

Fig. 1 Binding of albendazole (ABZ) to cytosolic proteins. Relationship between protein concentrations (optical density, O.D.) and ABZ concentrations (µg/mg protein) measured in 1 ml aliquots collected at different times during gel filtration (Sephadex G25) of *Fasciola hepatica* cytosolic proteins

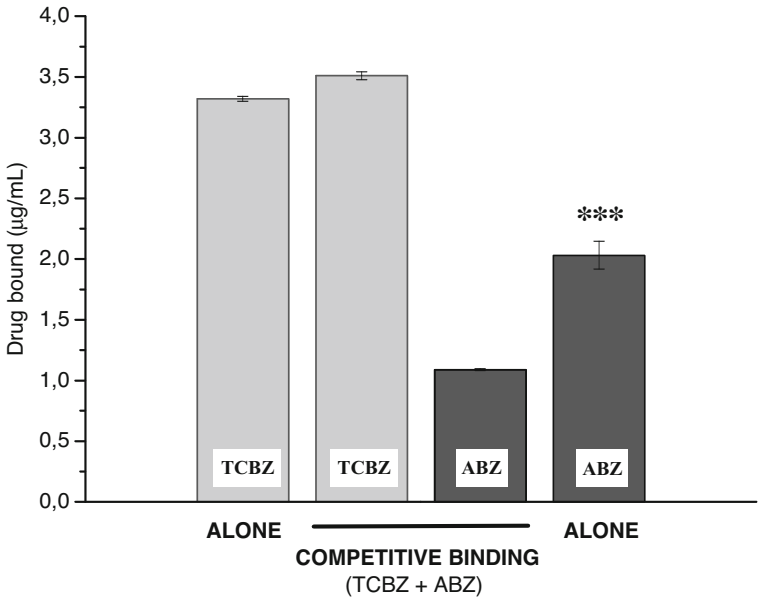

Fig. 2 Comparative binding of TCBZ (▒) and ABZ (▉) to cytosolic proteins from *Fasciola hepatica*, including the competitive binding between both molecules (TCBZ + ABZ). The data represents the amount of bound drug (µg/mL) eluted from a Sephadex G25 column and measured by HPLC. The values of ABZ alone bound to proteins are significantly different ($P<0.001$) from to those obtained after the elution of TCBZ + ABZ (competitive binding)

is still one of the most effective measures. Mechanisms of acquired anthelmintic resistance by parasites can be grouped into pharmacokinetic and pharmacodynamic-mediated mechanisms. Pharmacokinetic-mediated mechanisms include: increased drug inactivation, accelerated drug efflux and/or decreased drug uptake. The consequence would be to decrease drug accumulation within parasite's cells. Among the pharmacodynamic-mediated mechanism(s), the most relevant involve changes in the target molecule (i.e: structural modification, levels of synthesis and/or activation of downstream elements in pathways leading to a functional response) (Alvarez et al. 2005).

Different chemical interactions mediate drug binding to its target receptor. Furthermore, the pharmacological activity may also involve a "stereochemical correspondence" between the molecule and its receptor. BZD anthelmintics bind to their specific intracellular receptor (β-tubulin in nematodes) but also to other cytosolic proteins, which could be related to different cellular processes (related or unrelated to drug metabolism). This unspecific

Table 1 Comparative evaluation of the competitive binding of TCBZ and ABZ to cytosolic proteins from *Fasciola hepatica*

	TCBZ (µg/mL)		ABZ (µg/mL)	
	BOUND	FREE	BOUND	FREE
TCBZ alone	3,32±0,26 **(83%)**	0,69±0,11 **(17%)**		
ABZ alone			2,03±0,16 **(44%)**	2,61±0,02 **(56%)**
TCBZ + ABZ	3,51±0,043 **(87%)**	0,79±0,03 **(13%)**	1,09±0,01 **(23%)**	3,67±0,03 **(77%)**

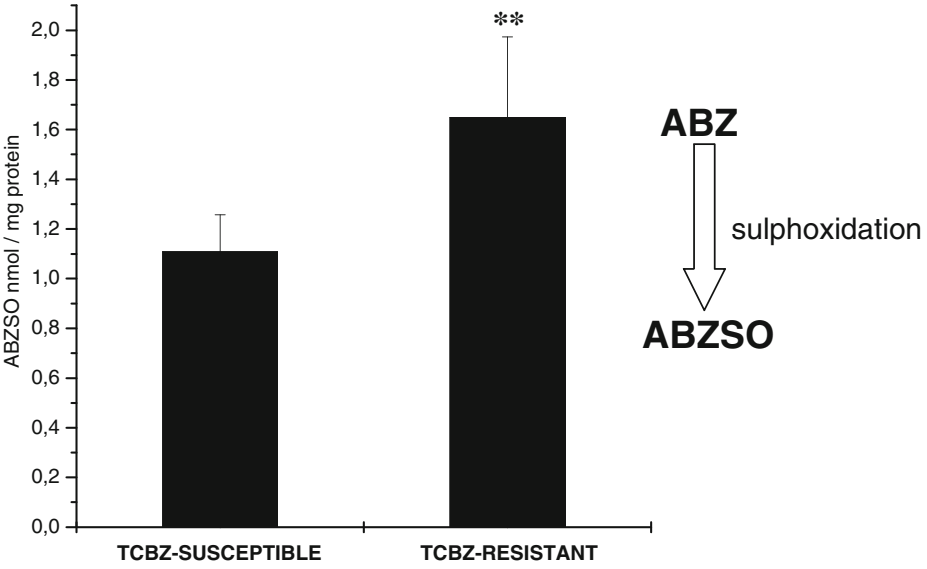

Fig. 3 Pattern of albendazole (ABZ) microsomal sulphoxidation in triclabendazole-susceptible and -resistant strains of *Fasciola hepatica*. The results show concentrations (nmol/mg.) of ABZ sulphoxide (ABZSO) formed after 60 min of incubation. Data represents mean ± S.D. ** Values are significantly different from those measured in a TCBZ-susceptible fluke at $P<0.005$

protein binding may have relevant implications for the desired pharmacological effect of the drug. Quantitative differences on BZD binding to cytosolic proteins were observed among different helminth species investigated (Solana et al. 2002a). Based on the assay conditions used in the current work, studies on the competitive binding to cytosolic proteins of F. hepatica demonstrated that tubulin (main target for the nematodicidal action of the BZDs) would not be the unique site for the trematodicidal action of TCBZ (Solana et al. 2002b). The fraction of TCBZ bound to cytosolic proteins of F. hepatica is not displaced by ABZ. There was no displacement even though ABZ concentrations were 3-fold higher than those of TCBZ (Fig. 2, Table 1). TCBZ may have greater affinity than ABZ by cytosolic proteins, including tubulin. As was pointed out by Brennan et al. (2007) information on TCBZ and ABZ molecular interaction with different parasitic structures may be relevant for improving the chemotherapy of fluke infections in domestic animals.

In contrast to the situation ocurring in parasitic nematodes, β-tubulin mutations were not correlated with TCBZ resistance in *F. hepatica* (Robinson et al. 2002; Ryan et al. 2008). The absence of alterations in a proposed target molecule for TCBZ action molecule (i.e. fluke β-tubulin), motivated a series of studies to assess the metabolic capacity in susceptible and resistant flukes. Those investigations demonstrated showed an enhanced ability to oxidize TCBZ oxidation pattern in resistant fluke specimens (Alvarez et al. 2005).

Helminth parasites such as *Ascaris lumbricoides* and *Moniezia expansa* seem to have an apparent lack of cytochrome P450 as a detoxification route. In these parasite species, oxidations are carried out exclusively by the FMO route (Precious and Barrett 1989). In *A. suum* and *M. expansa* the oxidative metabolism require NADH or NADPH as cofactors (Douch and Buchanan 1979). It has been shown that *F. hepatica* have significantly higher ability to oxidise ABZ into ABZSO and $ABZSO_2$ than nematode and cestode parasites (Solana et al. 2001). Besides, microsomes obtained from fluke specimens were able to

Fasciola hepatica	**ABZSO** *(nmoles/mg prot)*	***EE***	**Ratio** -/+
SUSCEPTIBLE *(Cullompton Strain)*	1.097	3.00	1.06
RESISTANT *(Sligo Strain)*	1.639	7.66	0.56

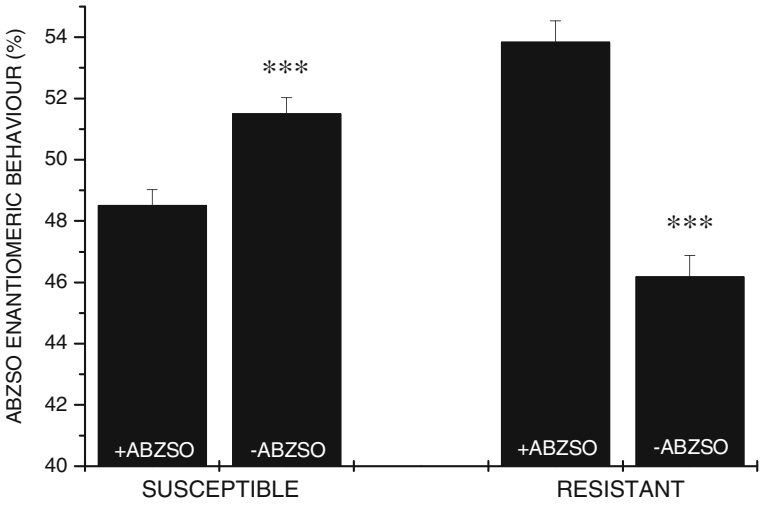

Fig. 4 Comparison of the albendazole sulphoxide (ABZSO) enantiomeric proportion produced after albendazole (ABZ) sulphoxidation by microsomes obtained from *Fasciola hepatica* susceptible and resistant to triclabendazole. The data illustrates the change in the pattern of ABZSO enantiomer formation. (***): The percentages of (-) ABZSO are statistically different ($P<0.001$) compared to those observed for (+) ABZSO. The inserted table summarizes the amounts of ABZSO formed, the enantiomeric excess (***EE***) and the ratio between (-) ABZSO and (+) ABZSO in both susceptible and resistant flukes

metabolise TCBZ into its sulphoxide metabolite (Mottier et al. 2004; Solana et al. 2003) and also to convert TCBZSO into $TCBZSO_2$ (Robinson et al. 2004). Previously, a higher capacity to oxidize TCBZ into TCBZO was observed in TCBZ-resistant compared to susceptible flukes. The assays described here were performed to gain further insight on the comparative metabolic ability of TCBZ-susceptible and resistant strains of *F. hepatica* over a chemically related compound. Interestingly, the sulphoxidation of ABZ was also higher in TCBZ-resistant flukes under the *in vitro* conditions described here. The absence of alterations in a proposed target molecule for TCBZ action molecule (i.e. fluke β-tubulin), motivated a series of studies to assess the mechanisms involved on the resistance to TCBZ in *Fasciola hepatica*. Altered drug uptake and metabolism may be more important than any changes to the presumed target molecule for TCBZ action (Brennan et al. 2007). The transtegumental influx/efflux balance for TCBZ and its main metabolites is altered in TCBZ-resistant flukes, which accounted for a reduced amount of active drug being recovered within the resistant flukes (Alvarez et al. 2005). In addition, the FMO-mediated TCBZ sulphoxidation was significantly higher in TCBZ-resistant than in TCBZ-susceptible flukes (Alvarez et al. 2005). Thus, the reduced drug/metabolite availability in addition to the enhanced BZD metabolism observed in adult resistant flukes, would severely limit the amount pharmacological active moieties reaching their molecular target within the parasite. However, despite its enhanced biotransformation in TCBZ-resistant flukes, ABZ is an alternative anthelmintic commonly used for fluke control under field conditions.

Enantioselectivity of metabolic products occurs when chiral metabolites are generated differentially (in qualitative or quantitative terms) from a single achiral substrate (Testa and

Mayer 1988). The FMO-mediated sulphoxidation of ABZ has been shown to be enantioselective in several animal species and this enzyme system is mainly involved in the production of the (+) enantiomeric form of ABZSO in mammals. Therefore, we speculate that the FMO system participate in the production of (+) ABZSO in *F. hepatica*. Based on this rationale, a higher FMO-mediated sulphoxidation of ABZ may have accounted for an increased production of (+) ABZSO in microsomes obtained from TCBZ-resistant fluke specimens (see Fig. 4). This finding agrees well with those showing that FMO substrates (i.e methimazole) markedly reduce the sulphoxidation of TCBZ in resistant liver flukes (Alvarez et al. 2005).

Finally, the results reported here contribute to the understanding of the relationship between drug metabolism in target parasites and the identification of possible mechanisms of resistance. In conclusion, the enhanced sulphoxidative capacity observed in the resistant strain is applicable to both ABZ and TCBZ metabolism. The enhanced drug metabolism in adult resistant flukes would severely limit the amount of TCBZSO or ABZSO reaching its target. On the other hand, while this altered oxidative metabolic pattern may cooperate to the resistance phenomenon, other pharmacodynamic-based mechanisms may be involved, which would explain why, although being chemically-related, ABZ remains efficacious against TCBZ resistant flukes in the field. The role of metabolic pathways and the activity and expression of efflux pumps affecting drug accumulation within the target parasite remain to be completely understood. Working on that direction may help to maintain the efficacy of ABZ and TCBZ before resistance to both molecules is widespread.

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