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RESISTANCE-INDUCED CHANGES IN TRICLABENDAZOLE TRANSPORT IN *FASCIOLA HEPATICA*: IVERMECTIN REVERSAL EFFECT

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ABSTRACT: Triclabendazole (TCBZ) and albendazole (ABZ) are flukicidal benzimidazole compounds extensively used in veterinary medicine. Although TCBZ has excellent activity against mature and immature stages of the liver fluke, *Fasciola hepatica*, ABZ action is restricted to flukes older than 12 wk. The intensive use of TCBZ has resulted in the development of resistance. To gain insight into the mechanisms of resistance to TCBZ, the ex vivo diffusion of TCBZ, TCBZ sulfoxide (TCBZSO, the active metabolite of TCBZ), and ABZ into TCBZ-susceptible and -resistant adult flukes was compared. TCBZ-susceptible (Cullompton) and -resistant (Sligo) flukes were incubated in Krebs-Ringer Tris buffer with either TCBZ, TCBZSO, or ABZ (5 nmol/ml) for 90 min. Drug/metabolite concentrations were quantified by high-performance liquid chromatography. All the assayed molecules penetrated through the tegument of both susceptible and resistant flukes. However, significantly lower concentrations of TCBZ and TCBZSO were recovered within the TCBZ-resistant flukes. In contrast, ABZ entrance into the susceptible and resistant flukes was equivalent. The influx/efflux balance for TCBZ, TCBZSO, and ABZ in susceptible and resistant flukes in the presence or absence of a substrate (ivermectin) of the drug transporter P-glycoprotein was assessed. The ivermectin-induced modulation of P-glycoprotein activity decreased TCBZ efflux from the resistant flukes. Higher concentrations of TCBZ and TCBZSO were recovered from the resistant liver flukes in the presence of ivermectin. Thus, an altered influx/efflux mechanism may account for the development of resistance to TCBZ in *F. hepatica*.

Fascioliasis, caused by the cosmopolitan trematode parasite *Fasciola hepatica*, is a very serious disease that produces considerable loss in sheep and cattle production systems worldwide (Boray, 1994). Benzimidazoles (BZDs) are broad-spectrum anthelmintic compounds widely used in human and veterinary medicine to control nematode, cestode, and trematode infections (McKellar and Scott, 1990). Only a few BZDs display activity against the *F. hepatica*. The halogenated derivative triclabendazole (TCBZ) is the most effective derivative, because of its excellent activity against adult and juvenile flukes (Boray et al., 1983). Consequently, it is the most widely used compund, and this extensive use has led to the selection and emergence of TCBZ-resistant fluke populations in several areas of the world (for review, see Fairweather, 2005). Because no new fasciolicides have been marketed recently, resistance could pose a serious threat if it becomes widespread (Coles, 2002). Albendazole (ABZ) is the only BZD carbamate recommended to control fascioliasis in domestic animals, despite its activity being restricted to flukes older than 12 wk (McKellar and Scott, 1990). TCBZ and ABZ are able to penetrate the tegument of *F. hepatica* by diffusion (Alvarez et al., 2004; Mottier, Virkel et al., 2004), and the fluke is able to sulfoxidate both drugs to their sulfoxide metabolites (TCBZ sulfoxide [TCBZSO] and ABZ sulfoxide [ABZSO], respectively) (Solana et al., 2001; Mottier, Virkel et al. 2004). The sulfoxide metabolites are the only pharmacologically active metabolites found in the bloodstream of treated animals (Marriner and Bogan, 1980; Hennessy et al., 1987). Interestingly, ABZ has been shown to be effective against TCBZ-resistant flukes (Coles and Stafford, 2001).

Parasites have several possible strategies to achieve drug resistance, including reduced uptake, active efflux, target modification, drug modification, drug sequestration, by-pass shunt, and substrate competition (Ouellette, 2001). BZD resistance in nematodes has been linked to the loss of high-affinity binding to tubulin (Lubega and Prichard, 1991b) and an alteration of the β-tubulin isoform pattern (Lubega and Prichard, 1991a), correlated with a conserved mutation at amino acid 200 (phenylalanine to tyrosine, F200Y) in tubulin isotype 1 (Kwa et al., 1994). Although experimental data support a microtubule-based action for TCBZ (for review, see Fairweather, 2005), it has been shown that the TCBZ-resistant phenotype is not associated with residue changes (specifically, the F200Y mutation) in the primary amino acid sequence of β-tubulin (Robinson et al., 2002). In seeking an alternative mechanism of resistance, previous work in our laboratory has demonstrated that the total amounts of TCBZ and TCBZSO (estimated as area under the concentration-time curve) recovered in TCBZ-resistant flukes were significantly lower (<50%) than those recorded in TCBZ-susceptible flukes (Alvarez et al., 2005). So, TCBZ-resistant flukes are exposed to lower concentrations of TCBZ and TCBZSO than TCBZ-susceptible flukes. This pivotal difference between the isolates may be by different mechanisms, but, most likely, it could be related to an altered drug influx/efflux and an increased capacity to oxidize TCBZ/TCBZSO to inert forms in TCBZ-resistant *F. hepatica*. The latter has been demonstrated by Alvarez et al. (2005), but it may not be sufficient in itself to fully explain the lower amounts of TCBZ/metabolites recovered from the resistant isolate. It has been suggested that additional mechanisms are in operation, mainly involving BZD molecule efflux carried out by drug transporters. P-glycoprotein (Pgp) is a member of the ATP-binding cassette (ABC) group of transporters that function as ATP-dependent efflux mechanism, enabling drugs to be expelled from cells (Gerlach et al., 1986). Overexpression of Pgp has been implicated in the resistance of nematodes to macrocyclic lactones (ivermectin [IVM] and moxidectin) (Pouliot et al., 1997; Xu et al., 1998), closantel, and BZD, although the exact nature of the role has yet to be established (Wolstenholme et al., 2004). Moreover, it has been shown that verapamil (a Pgp inhibitor) is able to partially reverse BZD resistance in *Haemonchus contortus* (Beugnet et al., 1997). An overexpression of these transporters in the

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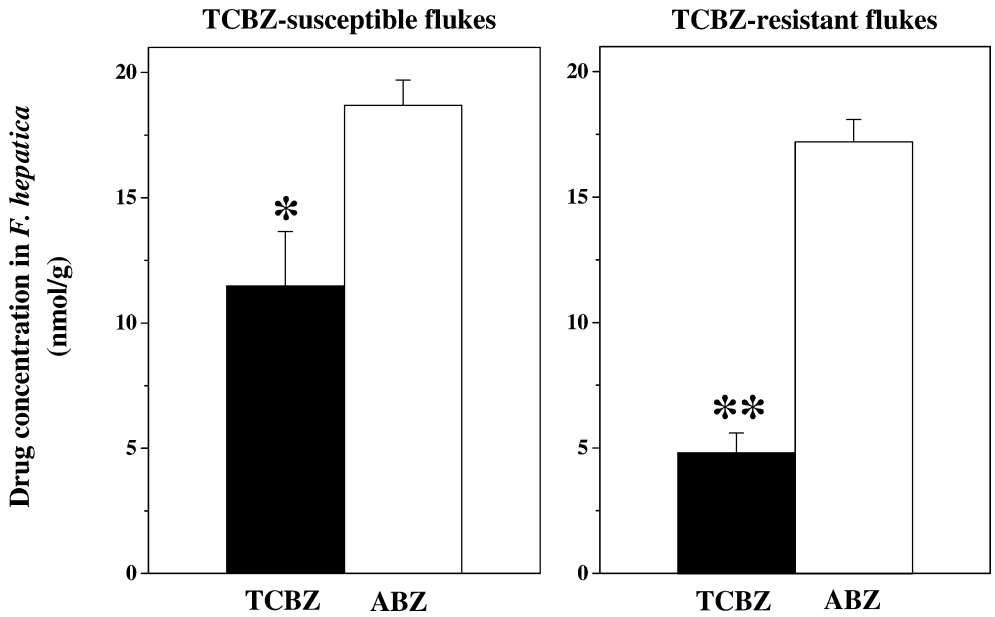

FIGURE 1. Comparative diffusion of triclabendazole (TCBZ) and albendazole (ABZ) into adult specimens of TCBZ-susceptible and -resistant *F. hepatica*. TCBZ concentrations are significantly lower than those of ABZ measured in TCBZ-susceptible ($*P < 0.001$) and -resistant ($**P < 0.0001$) flukes.

TCBZ-resistant flukes could explain the lower TCBZ and TCBZSO concentrations measured within TCBZ-resistant flukes.

To gain further insight into the mechanisms involved in resistance to TCBZ, the comparative ex vivo diffusion of TCBZ, TCBZSO, and ABZ into TCBZ-susceptible and -resistant adult flukes was determined. Differences in uptake between the 2 drugs in TCBZ-susceptible and -resistant flukes could be linked to differences in efficacy of ABZ and TCBZ against *F. hepatica*. Experiments were carried out in the presence or absence of an inhibitor of Pgp. The endectocide IVM was selected as the inhibitor because it has been shown to be a substrate for Pgp (Didier and Loor, 1996; Pouliot et al., 1997). Interactions between ABZ and TCBZSO also were studied in relation to their impact on drug uptake.

MATERIALS AND METHODS

Chemicals

Reference standards of TCBZ and TCBZSO (Novartis Animal Health, Basel, Switzerland), ABZ (Schering Plough, Kenilworth, New Jersey), and IVM (Bayer, Buenos Aires, Argentina) were used for the experimental assays. The solvents used for the chemical extraction and chromatographic analysis were high-performance liquid chromatography (HPLC) grade (J. T. Baker, Phillipsburg, New Jersey). Buffer salts ($NaHCO_3$, Na_2HPO_4, and CH_3COONH_4) were purchased from J. T. Baker.

Collection of parasite material

Ten parasite-free Corriedale-weaned lambs were each orally infected with 200 metacercariae of *F. hepatica* contained in a gelatin capsule. Five animals were infected with a TCBZ-susceptible isolate (named Cullompton) and the other 5 animals with a TCBZ-resistant isolate (named Sligo). For details of the history of the 2 isolates, see Robinson et al. (2004). Sixteen weeks after infection, the animals were stunned and exsanguinated immediately. Animal procedures and management protocols were approved by the Ethics Committee according to Animal Welfare Policy (Act 087/02) of the Faculty of Veterinary Medicine, Universidad Nacional del Centro de la Provincia de Buenos Aires (UNCPBA), Tandil, Argentina (http://www.vet.unicen.edu.ar) and to internationally accepted animal welfare guidelines (American Veterinary Medical Association, 2001).

Adult *F. hepatica* specimens (TCBZ-susceptible or -resistant) were recovered from the common bile ducts and the gall bladder of each lamb. The parasites were rinsed extensively with saline solution (0.9% [w/v] NaCl; 37 C) to remove bile and any adhering material.

Drug diffusion assays

The collected flukes (TCBZ-susceptible or -resistant) were maintained for 2 hr before starting the incubation process in a Krebs-Ringer Tris (KRT) buffer, pH 7.4, at 37 C (McCracken and Lipkowitz, 1990). Fluke specimens (approximately 0.1 g) of each isolate were incubated at 37 C in 1 ml of KRT buffer spiked with either TCBZ, TCBZ + IVM, TCBZSO, TCBZSO + IVM, ABZ, ABZ + TCBZSO, or ABZ + IVM (10 μl of each drug, previously dissolved in methanol). The final concentration of TCBZ, TCBZSO, and ABZ was 5 nmol/ml. The final concentration of IVM in the incubation medium was 1 μg/ml. Incubation time assayed was 90 min. For each fluke isolate, a single experiment was performed with 4 replicates for each drug compound. Blank samples containing parasite material and incubation medium without drug (spiked with 20 μl of methanol) or drug-spiked medium without parasite material were incubated for the same time interval. Once the incubation time had elapsed, the flukes were rinsed thoroughly with saline solution, blotted on coarse filter paper, and stored at −20 C until their preparation for HPLC analysis to measure drug concentrations. The parasite material was processed within 15 days of the incubation assays.

Sample preparation, extraction, and analytical procedures

HPLC analysis for TCBZ and its metabolites was carried out as described previously (Mottier, Moreno et al., 2004). With respect to ABZ and its sulfoxide metabolite, sample preparation and extraction procedures were carried out as described for the samples containing TCBZ and TCBZSO (Mottier, Moreno et al., 2004); however, HPLC analysis was performed as described by Mottier, Alvarez, and Lanusse (2003).

Drug concentrations are expressed as nanomoles per gram of *F. hepatica* wet weight. Drug-free *F. hepatica* material (0.1 g) was spiked with each drug (TCBZ, TCBZSO, ABZ, or ABZSO) to reach the following final concentrations: 0.5, 1.0, 2.5, 5.0, 10.0, and 20.0 nmol/g *F. hepatica* wet weight, and with the internal standard (IS) oxibendazole (OBZ) (10 μl, stock solution of 500 μM). Validation of the analytical

procedures for extraction and quantification of TCBZ, ABZ, and their sulfoxide metabolites from parasite material was carried out as described previously (Mottier, Alvarez, and Lanusse, 2003; Mottier, Moreno et al., 2004).

Analysis of data

The individual concentration values (expressed as nanomoles per gram of *Fasciola hepatica* wet weight) are presented as mean ± SD. Statistical analysis of the data was performed using Student's *t*-test to compare drug concentrations obtained inside TCBZ-susceptible and -resistant liver flukes incubated with a single drug or a drug combination. The statistical analysis (regression analysis and comparison of means) was performed using Instat 3.0 (GraphPad Software Inc., San Diego, California). When an analysis of variance was used and a significant *F* value was obtained, Tukey's range test was performed to indicate order of significance.

RESULTS

The accumulation of TCBZ into TCBZ-resistant *F. hepatica* was significantly lower (58%) than that observed for the TCBZ-susceptible flukes: 4.8 and 11.5 nmol/g, respectively ($P < 0.001$) (Fig. 1). A similar pattern was observed for the active metabolite TCBZSO, where concentrations achieved inside the TCBZ-resistant isolate were significantly lower (47%) compared with those measured in the susceptible parasites (10.4 and 15.3 nmol/g, respectively; $P < 0.005$) (Fig. 2). In contrast, the accumulation of ABZ into the TCBZ-susceptible and -resistant flukes was equivalent (18.7 and 17.2 nmol/g, respectively) (Fig. 1). Furthermore, the accumulation of ABZ by TCBZ-susceptible liver flukes was significantly higher (63%) ($P < 0.001$) than for TCBZ (Fig. 1). This difference becomes even greater if the concentration profiles of the 2 BZD molecules are compared in the TCBZ-resistant flukes: the transfer of ABZ was 258% greater than that of TCBZ (Fig. 1).

The comparative diffusion of ABZ into TCBZ-susceptible and -resistant flukes is shown in Figure 1. As mentioned, there were no significant differences in the ABZ concentrations measured in the TCBZ-susceptible and -resistant parasite. Nor were any significant differences observed between the amounts of ABZ measured in the TCBZ-susceptible flukes after coincubation of ABZ with either TCBZSO (17.2 nmol/g) or IVM (20.0 nmol/g) compared with the concentration achieved when the parasites were exposed to ABZ alone (18.7 nmol/g). However, ABZ concentrations in TCBZ-susceptible *F. hepatica* when TCBZSO or IVM was available in the incubation medium was significantly higher ($P < 0.05$) than that reached in the TCBZ-resistant flukes (14.3 and 17.5. nmol/g, respectively).

Higher concentrations of ABZSO (the sulfoxide metabolite of ABZ) were measured in the TCBZ-resistant flukes (~2.7 nmol/g) compared with the susceptible flukes (~1.2 nmol/g).

TCBZSO concentrations measured in the TCBZ-susceptible and -resistant flukes when they were coincubated with ABZ were 17.2 ± 1.0 and 11.8 ± 1.3 nmol/g, respectively.

The concentrations (mean ± SD) of TCBZ and TCBZSO, incubated alone or coincubated with IVM for 90 min and measured in TCBZ-resistant *F. hepatica*, are shown in Figure 3. When IVM was added to the incubation medium, the concentration of TCBZ recovered inside the TCBZ-resistant flukes was significantly higher (27%) than that measured when IVM was absent (6.1 and 4.8 nmol/g, respectively; $P < 0.05$). This was also true for TCBZSO in the TCBZ-resistant isolate: 13.3 and 10.4 nmol/g, respectively ($P < 0.05$). Furthermore, when the flukes were coincubated with IVM, equivalent concentrations of TCBZSO were achieved in both the TCBZ-susceptible and -resistant isolates (~13 nmol/g) (Figs. 2, 3).

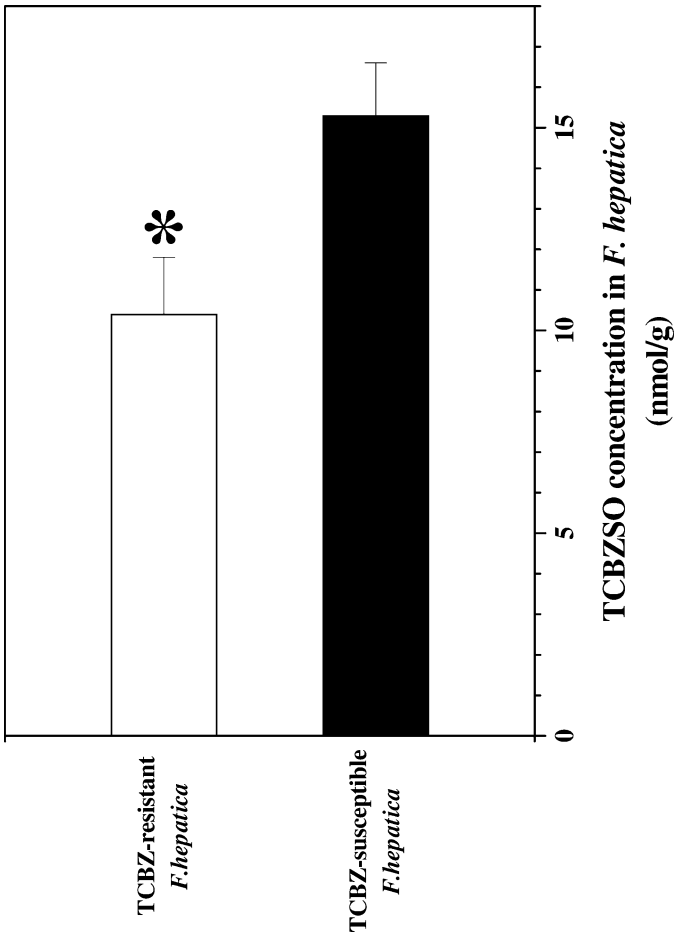

FIGURE 2. Assessment of triclabendazole sulfoxide (TCBZSO) transfer into adult specimens of TCBZ-susceptible and -resistant *F. hepatica*. TCBZSO concentrations measured in TCBZ-resistant *F. hepatica* are significantly lower (*$P < 0.005$) than those measured in the TCBZ-susceptible flukes.

DISCUSSION

One aim of the present study was to investigate the mechanism of resistance to TCBZ in *F. hepatica*, particularly in relation to drug influx and efflux. Another aim was to try to understand why ABZ, a related BZD molecule, has activity against TCBZ-resistant fluke. This is unusual, because it is thought that resistance to one member of a group of drugs (such as the BZDs) confers resistance to other members of the same group (McKellar and Jackson, 2004). The study set out to determine whether the differential efficacy of ABZ and TCBZ could be linked to differences in drug influx/efflux between TCBZ-susceptible and -resistant flukes. The results are discussed in terms of these processes.

The uptake of ABZ was greater than TCBZ in TCBZ-susceptible flukes; the difference was even more marked in TCBZ-resistant flukes, with a 258% difference. This difference may be linked to the higher lipophilicity of ABZ. Thus, its log P

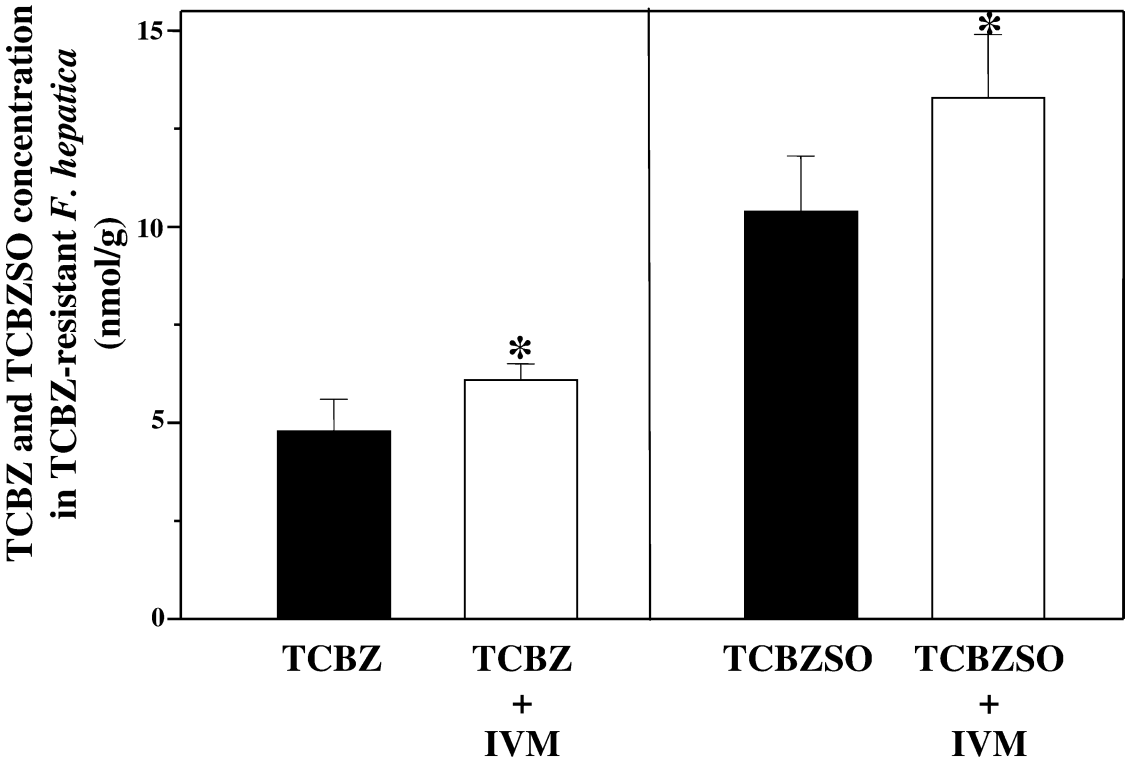

FIGURE 3. Effect of ivermectin (IVM) on the amounts of triclabendazole (TCBZ) and triclabendazole sulfoxide (TCBZSO) recovered from adult specimens of TCBZ-resistant *F. hepatica*. Drug concentrations measured in the resistant flukes incubated in the presence of IVM are significantly higher (*$P < 0.05$) than those measured in TCBZ-resistant *F. hepatica* incubated in its absence.

value is 3.82, one of the highest values obtained for BZD methylcarbamates (Mottier, Alvarez et al., 2003) compared with that of 3.48 for TCBZ (Mottier, Virkel et al., 2004). A similar difference between the 2 drugs has been obtained for another susceptible isolate (from DILAVE, Instituto Miguel C. Rubino, Montevideo, Uruguay), indicating that it is a more widespread phenomenon (Alvarez et al., 2004). In the present study, the uptake of ABZ was similar in both isolates. In contrast, significantly lower (~50%) concentrations of TCBZ and TCBZSO were recovered in TCBZ-resistant compared with -susceptible flukes. The difference in uptake may not in itself explain the high degree of resistance in the Sligo isolate observed under field conditions (Coles and Stafford, 2001). Nevertheless, the result confirms a previous study carried out on the same isolates, but with different sheep and with different batches of cysts (Alvarez et al., 2005). This suggests that the difference is genuine and is related to the TCBZ-resistant isolate.

An alternative explanation for the resistance mechanism involves altered drug metabolism in TCBZ-resistant flukes. Increased metabolism of drugs by these flukes could lead to the lower drug concentrations observed. Susceptible flukes have been shown to possess the ability to metabolize both ABZ and TCBZ to their respective sulfoxide and sulfone metabolites (Solana et al., 2001; Mottier, Virkel et al., 2004; Robinson et al., 2004). Moreover, it has been shown that TCBZ-resistant flukes have an enhanced capacity to sulfoxidate the 2 drugs compared with their susceptible counterparts (Robinson et al., 2004; Solana et al., 2004; Alvarez et al., 2005). The higher ABZSO concentrations measured in the TCBZ-resistant flukes in the current ex vivo diffusion assays are in agreement with those findings, although this apparent increase does not seem to be correlated with ABZ resistance.

Drug accumulation in parasites depends on the balance between drug influx and drug efflux. Equivalent concentrations of ABZ were measured in both isolates, indicating that BZD influx is not altered in TCBZ-resistant flukes. The same is true for $TCBZSO_2$, the inactive and more hydrophilic metabolite of TCBZ (Alvarez et al., 2005). Therefore, a selective process of efflux for TCBZ and TCBZSO may be involved. Because the entry of TCBZSO into the liver fluke occurs principally by passive diffusion across its tegument (Mottier et al., 2006), this precludes changes in any active uptake mechanism in TCBZ-resistant flukes. It remains possible that lower TCBZ concentrations are regulated by efflux mechanisms in TCBZ-resistant fluke.

Drug efflux from cells is mediated by ATP-dependent transmembrane transporters such as Pgp (Jones and George, 2005). Pgp genes have been identified in nematodes, i.e., at least 14 in *Caenorhabditis elegans* (Lincke et al., 1992), at least 7 in *Haemonchus contortus* (Sangster, 1994; Kwa et al., 1998; Xu et al., 1998; Sangster et al., 1999), and 2 in *Onchocerca volvulus* (Kwa et al., 1998; Huang and Prichard, 1999). In trematodes, 2 genes encoding ABC proteins have been identified in the human blood fluke *Schistosoma mansoni* (Bosch et al., 1994), and an ABC transporter has been described for *F. hepatica* (Reed et al., 1998). Although the complete sequence has not been determined, the N-terminal two-thirds of the coding sequence indicate that this gene is likely to be a Pgp. The avermectin/milbemycin anthelmintics interact with Pgp, and IVM can be used as a multidrug resistance (MDR)-reversing agent (Pouliot et al., 1997). It has been shown that *H. contortus* re-

sistant to IVM possessed an increased level of Pgp expression (Xu et al., 1998) and that the coapplication of verapamil (an MDR-reversing agent) increased the efficacy of IVM and moxidectin against resistant strains of *H. contortus* in jirds (Xu et al., 1998; Molento and Prichard, 1999). Similarly, an overexpression of Pgp could explain the lower concentrations of TCBZ and TCBZSO measured in TCBZ-resistant *F. hepatica*.

Is there any evidence that TCBZ and TCBZSO serve as substrates for Pgp? Specific binding between BZD molecules and Pgp has been demonstrated in human cells (Nare et al., 1994), and it has been shown that verapamil (a Pgp inhibitor) is able to partially reverse BZD resistance in *H. contortus* (Beugnet et al., 1997). However, Merino et al. (2002) reported that ABZ is neither a substrate nor inhibitor of Pgp. The measurement of similar levels of ABZ in TCBZ-susceptible and -resistant flukes in the current study would support this last fact. In contrast, TCBZ and TCBZSO may be substrates/inhibitors of Pgp, because of the lower levels of the drugs in TCBZ-resistant flukes. The difference was maintained after coincubation of TCBZSO with ABZ, even though the levels of ABZ remained the same in the 2 isolates. This indicates that no competition for Pgp exists between ABZ and TCBZSO. More conclusive evidence was obtained from the experiments involving coincubation with IVM. Equivalent concentrations of ABZ were obtained in both isolates, whereas concentrations of TCBZ and TCBZSO in TCBZ-resistant flukes were higher than those measured in the absence of IVM. The results support a previous study (Alvarez et al., 2005) in signifying a role for Pgp in the mechanism of resistance to TCBZ.

The results reported here confirm those previously reported by Alvarez et al. (2005), where TCBZ and TCBZSO accumulation in resistant *F. hepatica* was lower (about 50%) compared with the TCBZ-susceptible isolate. An IVM-induced inhibition of Pgp activity may account to explain the enhancement of TCBZ and TCBZSO concentrations (30%) observed in the resistant *F. hepatica*. However, this phenomenon by itself does not fully explain the high level of resistance displayed by the Sligo isolate under field conditions (Coles and Stafford, 2001). It is likely that an additional mechanism(s) of drug resistance may be involved in the high degree of resistance to TCBZ observed in the Sligo isolate of *F. hepatica*. Thus, further work is required to elucidate the relationship between drug accumulation and parasite lethality in both strains of *F. hepatica*, which could help to interpret the mechanism of resistance.

In conclusion, the present results have provided further support for the involvement of Pgp-linked drug efflux pumps in the mechanism of resistance to TCBZ, although the idea needs to be validated by molecular studies. The study also has aided in understanding why a related BZD such as ABZ possesses activity against TCBZ-resistant flukes. However, the picture is incomplete, and the relative contributions of drug uptake and metabolism to resistance and interactions between drugs remain to be clarified further.

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