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Drug transport mechanisms in helminth parasites: Passive diffusion of benzimidazole anthelmintics

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Abstract

Anthelmintic molecules must reach their receptors inside target parasites to exert the pharmacological effect. Available data suggest that the main route of entry of antiparasitic drugs into helminth parasites would be through their external surface. However, it is unclear if trans-tegumental/cuticular penetration is the most important way of entry of benzimidazole (BZD) anthelmintics into their target parasites compared to oral ingestion. The relative involvement of active and passive transport mechanisms has not been defined. The goal of the work reported here was to determine the main processes involved in the entry of BZD anthelmintic molecules into the three main classes of helminth parasites. Adult specimens of *Moniezia benedeni* (cestode), *Fasciola hepatica* (trematode) and *Ascaris suum* (nematode) were incubated in Kreb's Ringer Tris buffer (pH 7.4, 37 °C) (1 g parasite/10 ml incubation medium) for 15, 45, and 90 min, respectively, in the presence of a concentration gradient of either fenbendazole (FBZ), oxfendazole or triclabendazole sulphoxide (TCBZSO) (1–30 mol/ml, $n = 4$). Dead helminth specimens were also incubated with the same drug concentration gradient. Specimens of *F. hepatica* with the oral route closed off by ligation were incubated with TCBZSO in the presence or absence of bovine serum albumin. After the incubation time elapsed, samples of parasite material were chemically extracted and prepared for high performance liquid chromatography analysis to measure drug/metabolite concentrations. Equivalent drug concentrations were measured within ligated and non-ligated liver flukes, demonstrating that BZD do mainly penetrate by trans-tegumental diffusion. The higher the concentration of BZD molecules in the incubation medium, the greater their concentration recovered within the helminth parasites. High correlation coefficients (>0.98) were obtained between initial drug concentration in the incubation medium and those measured inside the nematode, cestode, and trematode parasites. FBZ concentrations recovered from tissues of dead cestodes/nematodes over time were significantly greater compared to those measured in living parasites. These differences in drug diffusion may be related to the morphological/functional properties of the parasite's external surfaces. The outcome of the work reported here indicates that passive drug transfer through the external helminth surface is the main transport mechanism accounting for BZD accumulation into target parasites.

Index Descriptors and Abbreviations: Benzimidazole anthelmintic; Helminth parasites; Drug transport mechanisms; Cuticle; Tegument; Passive diffusion

1. Introduction

Helminth parasites infect a quarter of the world's total population and are a major cause of morbidity (Colley et al., 2001). Moreover, helminth infections are the most important cause of productivity losses in livestock worldwide (Waller, 2003). Benzimidazoles (BZD), imidazothiazoles, and the macrocyclic lactones (ML) (avermectins/milbemycins) are the most important chemical groups used to control parasitic infections in domestic animals. The pharmacological activity of BZD, imidazothiazoles, and ML is based on their affinity for specific receptors located inside the target parasite: β-tubulin, acetylcholine-gated channels and glutamate-gated chloride channels, respectively. The pharmacokinetic behaviour of an

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anthelmintic drug involves the time course of drug absorption, tissue distribution, metabolism, and excretion, which in turn determines the concentration of the anthelmintic drug at the site of parasite location. Furthermore, anthelmintics need to reach their specific receptor within the target parasite to exert their action. Consequently, drug entry into a parasite is critical for anthelmintic efficacy.

Anthelmintic drugs can reach target helminth parasites by either oral ingestion (from the host's blood, and/or gastrointestinal contents) or by diffusion through the external surface, named cuticle in nematodes or tegument in cestodes and trematodes, or by some combination of both routes (Thompson et al., 1993; Thompson and Geary, 1995). The available data suggest that different chemical substances, including some anthelmintic drugs, would be mainly taken up through the external surface of cestodes (Alvarez et al., 1999; Mottier et al., 2003b), trematodes (Alvarez et al., 2000, 2001, 2004; Bennett and Köhler, 1987) and nematodes (Alvarez et al., 2000, 2001; Cross et al., 1998; Ho et al., 1990; Sims et al., 1992) as opposed to oral ingestion. In both cases (tegument/cuticular or intestinal entry), the anthelmintic molecules need to pass through cell membranes to reach the biophase at the specific receptor location site.

Drug molecules move across cell membranes either by passive diffusion or specialised transport processes. In the passive diffusion process, the membrane behaves as an inert lipid–pore boundary, and drug molecules traverse this barrier either by diffusion through the lipoprotein region or, alternatively, filtering through aqueous pores (channels) without the cellular expenditure of energy if they are of sufficiently small size. The entry of a drug into the parasite may depend on the diffusion surface, the concentration gradient across the membrane, the pH/pK relationship and the lipophilicity of the drug molecule. Specialised transport is another potential mechanism of drug entry into target parasites and is relatively selective toward the chemical nature of the substance and requires direct expenditure of energy (Baggot, 1982). If the entry of an anthelmintic to a target parasite is mediated by specialised transport (a saturable process), the time of drug exposure to the parasite would be critical to the final anthelmintic effect. On the other hand, if passive diffusion is a main mechanism of entry of a lipophilic drug, drug concentration, and the time would be both relevant for drug action, and the restrictions imposed by tegumental/cuticular lipid barriers will probably be similar to those of any cellular membrane. Therefore, knowledge of the mechanism of drug entry into target parasites is pivotal to understand the drug–parasite relationship and to optimize drug use.

Although the accumulated information would indicate that passive diffusion rather than specialised transport is implicated in drug entry into parasites, there is not available data that specifically demonstrates the mechanism of entry of BZD molecules into target parasites. Thus, the role of the cuticular/tegumental structure on the process of drug uptake by helminth parasites requires further characterization. Considering the well established structural/functional differences between the cestode/trematode tegument and the nematode cuticle (Halton, 2004; Thompson and Geary, 1995), the goals of the current experimental work were: (a) to elucidate the ex vivo relationship established between drug concentrations in the incubation medium and those recovered from the incubated parasite, using *Moniezia benedeni* (cestode), *Fasciola hepatica* (trematode), and *Ascaris suum* (nematode) as model helminths; and (b) to determine the relative contribution of the trans- tegumental/cuticular and oral routes using intact, living and dead adults specimens of the three classes of parasites under ex vivo conditions. Fenbendazole (FBZ) (a BDZ methylcarbamate molecule used as nematodicidal/cestodicidal drug) was chosen as a model of BZD drug for the diffusion assays into *A. suum*, *M. benedeni*, and *F. hepatica*, due to its high lipophilicity (Mottier et al., 2003b) and capacity to cross through the external surfaces of parasites. Oxfendazole (OFZ), another BZD molecule assayed here, is the pharmacological less active sulphoxide metabolite of FBZ with a lower lipophilicity than its parent drug. On the other hand, the entry of triclabendazole sulphoxide (TCBZSO) into liver flukes was also evaluated, as this molecule is the only active sulphoxide metabolite of triclabendazole (TCBZ) (the most widely used flukicidal drug) found in plasma after a conventional TCBZ treatment.

2. Materials and methods

2.1. Chemicals

Reference standards (97–99% pure) of FBZ and OFZ were provided by Rhone Merieux (Lyon, France), TCBZSO by Novartis Animal Health (Basel, Switzerland), and mebendazole (MBZ) (used as internal standard, is) by Schering Plough (Kenilworth, USA). All the solvents (acetonitrile and methanol) used during the extraction and drug analysis were HPLC grade and purchased from Sintorgan (Buenos Aires, Argentina). Water was double distilled and deionized using a water purification system (Simplicity, Millipore, Brazil). Buffer salts (KCl, $CaCl_2{\cdot}2H_2O$, $MgCl_2{\cdot}7H_2O$) and cupric sulphate ($CuSO_4$) were purchased from Merck (Germany) and Baker Inc. (Phillipsburg, USA), respectively. Bovine serum albumin (BSA), NaCl, Tris (hydroxymethyl) aminomethane hydrochloride (Trizma hydrochloride) and bicinconinic acid were purchased from Sigma (St. Louis, MO, USA).

2.2. Collection of parasite material

Specimens of *M. benedeni* and *A. suum* were collected from the small intestine of untreated cattle and pigs, respectively, killed at the local abattoir. To obtain *F. hepatica* specimens, eight (8) parasite-free Corriedale sheep were infected with 200 metacercariae of *F. hepatica*, given in a gelatine capsule by the oral route. Sixteen (16) weeks after infection, sheep were killed by captive bolt plus exsanguina-

tion, following internationally accepted animal welfare guidelines. 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 (AVMA, 2001). To recover adult specimens of *F. hepatica* from the liver, common bile ducts and the gall-bladder of each sheep were removed and opened. The collected *M. benedeni*, *A. suum*, and *F. hepatica* specimens were rinsed extensively with physiological saline solution (37 °C) to remove intestinal debris, bile and/or adhering materials.

2.3. Drug diffusion assays

The collected parasites were maintained for two hours before starting the incubation in a Kreb's Ringer Tris (KRT) buffer (pH 7.4) at 37 °C (McCracken and Lipkowitz, 1990). The incubations contained parasite material and KRT medium in a 1:10 proportion. The incubation assays for each drug concentration and each parasite species was conducted in four (4) replicates. Blank samples containing parasite material and incubation medium without drug, and drug-spiked medium without parasite material were incubated for the same time intervals. Following incubation, the parasite samples were rinsed thoroughly with physiological saline solution, blotted on coarse filter paper and stored at −20 °C until processed for high performance liquid chromatography (HPLC) analysis. The parasite material was processed shortly after the incubation assays.

The concentration gradient used for the incubation assays (1–30 nmol/ml) is based on pharmacological relevant concentrations obtained from previously reported studies where the gastrointestinal concentrations of BZD compounds were measured after conventional treatments in ruminant species (Alvarez et al., 1999; Hennessy et al., 1993a,b; Sánchez et al., 1997).

2.3.1. Moniezia benedeni

The proglottides of this cestode parasite (0.5 g) were incubated at 37 °C in 5 ml KRT buffer containing varying concentrations of either FBZ or its sulphoxide metabolite OFZ (1, 2.5, 5, 10, and 15 nmol/ml). The incubation time used to evaluate the diffusion of these BZD anthelmintics into *M. benedeni* was 15 min.

2.3.2. Fasciola hepatica

Adult flukes (approximately 0.1 g) were incubated during 45 min at 37 °C in 1 ml KRT buffer. The trematode parasites were incubated in the presence of different FBZ (1, 2.5, 5, 10, and 15 nmol/ml) and TCBZSO (1, 2.5, 5, 10, and 20 nmol/ml) concentrations. To determine the relative contribution of the transtegumental vs gastrovascular entry of FBZ and TCBZSO, absorption kinetics studies were performed using ligated and non-ligated adult living liver flukes (drug concentration 5 nmol/ml, 45 min of incubation). In these studies, ligatures were tied, using human hair, closing the oral sucker of the liver flukes. The ligatures were as tightly as possible without damaging the tegument. Complementary studies were run to assess tegument integrity: These studies showed that the dye Evan's Blue (0.01%) did not infiltrate the region around the ligature during the following incubation. Liver flukes tend to regurgitate their gastrovascular content during incubations. However, during the incubation period it could be observed that the gastrovascular content present inside the parasite was constant. As a consequence, the effectiveness of the ligatures to prevent TCBZSO/FBZ entry by the oral route was confirmed. Simultaneously, ligated and non-ligated adult flukes were incubated with TCBZSO (5 nmol/ml KRT, 45 min) in the presence and absence of BSA (30 mg/ml KRT, a physiological concentration of albumin in sheep plasma).

2.3.3. Ascaris suum

Intact adult females of the nematode *A. suum* (approximately 1 g) were incubated at 37 °C in 10 ml of the KRT buffer containing FBZ at the following different final concentrations: 1, 2.5, 5, 10, 20, and 30 nmol/ml. Higher concentrations of FBZ (20 and 30 nmol/ml) were used for the case of *A. suum*, as the external surface of this nematode parasite is structurally more complex, and lower FBZ concentrations were expected to be recovered within the parasite. The incubation time used to evaluate the diffusion of FBZ into *A. suum* was 90 min.

To rule out the existence of any oral ingestion and active transport (properties of living parasites/tissues) of the compounds under study, the same incubation procedures were done using dead (frozen–unfrozen) cestode, nematode, and trematode parasite material.

The differences in incubation time used for the three parasites are related to their external surface structure. That is, the more complex the structure, the longer the required incubation time. The incubation times assayed were chosen according to previous results obtained in our laboratory (Alvarez et al., 1999, 2000) where the results of the ex vivo diffusion of BZD compounds into cestode, trematode, and nematode parasites demonstrated that, at these selected incubation times (15, 45, and 90 min for *M. benedeni*, *F. hepatica*, and *A. suum*, respectively), the entry of the BZD molecules was linear. The highest drug concentration used in the current experiments was limited by the aqueous solubility of BZD compounds.

2.4. Measurement of parasite protein concentrations

Protein content of the three parasites was estimated and to facilitate understanding of comparative results, the final concentration values for the different drugs assayed are expressed as nmol/100 mg protein. The determination of parasite protein concentrations was adapted from that described by Smith et al. (1985).

2.5. Measurement of drug concentrations

Drug-free cestode, trematode, and nematode material (0.5, 0.1, and 1 g, respectively) was spiked with the internal standard (is) compound, MBZ (10 µl, stock solution of 500 µM) and each molecule used in the incubation assays (FBZ, OFZ or TCBZSO) to reach the following final concentrations: 1, 2.5, 5, 10, 20, and 30 nmol/g parasite protein. After 5 min, the parasite material was homogenised (15 s, at 4 °C) (Ultraturrax, T 25, Ika Works, Labortechnik, Wilmington, NC, USA). The parasite homogenate was mixed with 1.5 ml acetonitrile (three times) and shaken (multi-tube vortexer, VWR Scientific Products, West Chester, PA, USA) over 5 min to extract the drug analyte(s) present in the sample, and then centrifuged (Jouan, BR 4i Centrifuge, Saint Herblain, France) to allow phase separation (2000*g*, 10 min, 10 °C). The final collected acetonitrile phase (4.5 ml) was concentrated to dryness in a vacuum concentrator (Speed-Vac, Savant, Los Angeles, CA, USA), and then reconstituted with 150 µl mobile phase. Fifty microliters of each solution were injected into the chromatographic system. Spiked parasite material samples were analysed by HPLC as described previously (Mottier et al., 2003a, 2004a) to measure the concentrations of each drug assayed. Blank unspiked parasite samples were prepared with the same extraction procedure. Briefly, experimental and spiked parasite samples were analyzed to measure the concentrations of each drug by HPLC using a model 10 A system (Shimadzu, Kyoto, Japan). The extraction efficiency of the different analytes from parasite material samples, expressed as absolute recovery (over 85%) with a coefficient of variation (CV) of $\leqslant 20\%$. The molecules under study were identified by comparison with the retention times of pure drug standards, which were used to prepare standard solutions to construct the calibration lines for each analyte in the parasite material analysed. The linear regression lines for each analyte (in the range of concentrations used for the incubation assays, with triplicate determinations) showed correlation coefficients greater than 0.99. The concentrations of each analyte were quantified by comparison of the chromatographic peak area of each analyte with that obtained for the internal standard, using the Class LC 10 Software (Shimadzu, Kyoto, Japan) on an IBM 486-AT computer.

2.6. Analysis of the data

The individual concentration values (expressed as nmol/100 mg protein) are reported as mean ± standard deviation (SD). The statistical analysis of the data was performed as follows: (a) Student's *t* test was used to compare drug concentrations obtained in the different living and dead parasite tissues, and FBZ and OFZ concentrations achieved inside *M. benedeni*; (b) the comparison of FBZ concentrations achieved in *M. benedeni*, *F. hepatica* and *A. suum* at the different drug concentrations assayed, and TCBZSO concentrations measured in ligated/non-ligated/BSA was performed by analysis of variance (ANOVA). The statistical analysis (regression analysis and comparison of mean values) was performed using the Instat 3.0 Software (Graph Pad Software, San Diego, CA, USA). When ANOVA was employed and a significant *F* value was obtained, Tukey's range test was performed to indicate order of significance.

3. Results

Protein concentrations ($n = 10$) in *M. benedeni*, *F. hepatica*, and *A. suum* were 31.2 ± 4.4, 91.9 ± 11.2, and 102.3 ± 17.3 mg/g parasite material, respectively.

The accumulation of BZD anthelmintics inside the incubated parasites occurred in favour of a concentration gradient, regardless if they were dead or alive (Figs. 1–3). The higher the drug concentration in the incubation medium, the greater the amount recovered from the tissues of the three parasites. High correlations between drug concentrations in the incubation medium and those measured within the three types of helminth parasites, both living and dead, were obtained after linear regression analyses ($r \geqslant 0.98$) (Figs. 4–6).

BZD molecules concentrate inside not only on the viable parasites but also on the dead worms, although important differences were observed between them. BZD concentrations (mean ± SD) measured in living and dead *M. benedeni* (FBZ), *A. suum* (FBZ), and *F. hepatica* (TCBZSO), at the different drug concentrations assayed are shown in Figs. 1–3, respectively. The amounts of FBZ recovered from the tissues of the dead cestodes and nematodes over time were

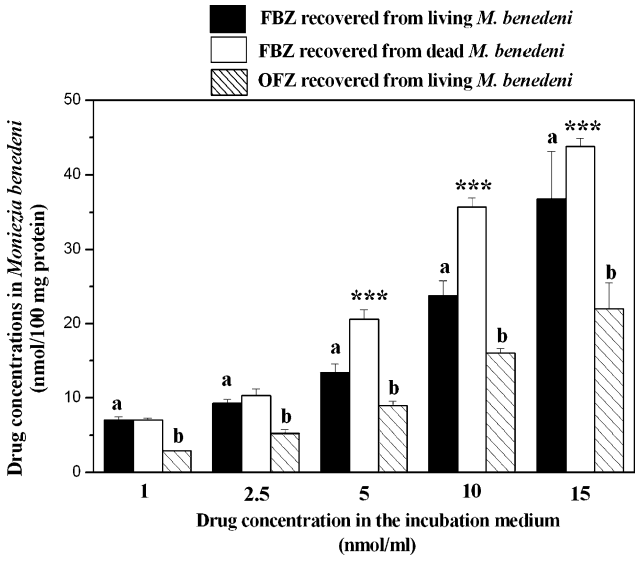

Fig. 1. Comparative ex vivo diffusion of fenbendazole (FBZ) and oxfendazole (OFZ) into living and dead specimens of *Moniezia benedeni* (cestode). Results represent mean concentrations (±SD, $n = 4$) of each compound measured in *M. benedeni* after a 15-min incubation in a gradient drug concentrations. Concentration values obtained for FBZ in living parasites were significantly lower that those measured in the dead specimens at $^{***}p < 0.0005$, and ^{a,b} greater than those achieved by OFZ at $p < 0.01$.

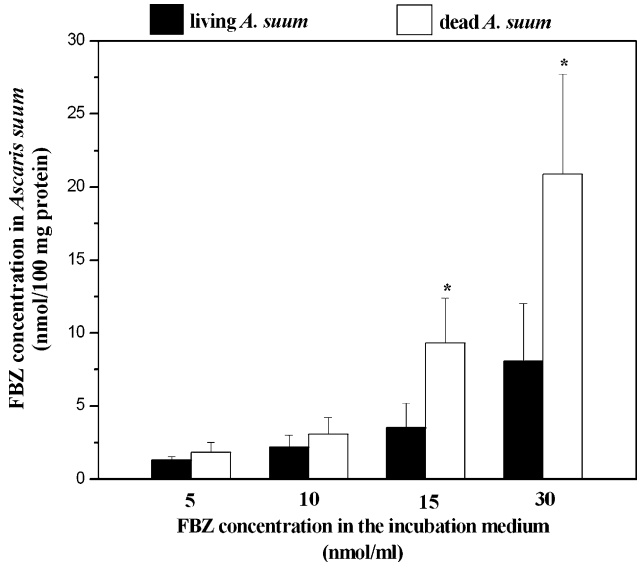

Fig. 2. Comparative ex vivo diffusion of fenbendazole (FBZ) into living and dead *Ascaris suum* (nematode). Results represent mean FBZ concentrations (±SD, $n = 4$) measured in *A. suum* after a 90-min incubation in a drug gradient concentration. Concentration values obtained for FBZ in living parasites were significantly lower that those measured in the dead ones at $*p<0.05$.

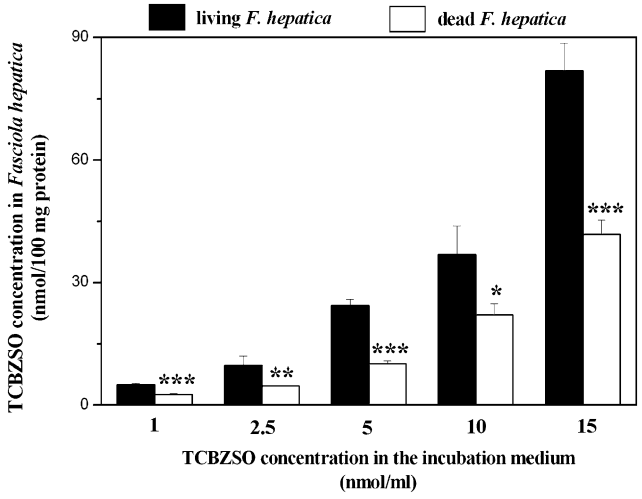

Fig. 3. Comparative ex vivo diffusion of triclabendazole sulphoxide (TCBZSO) into living and dead *Fasciola hepatica* (trematode). Results represent mean TCBZSO concentrations (±SD, $n = 4$) measured in the liver flukes after a 45-min incubation in a drug gradient concentration. Concentration values obtained for TCBZSO in living parasites were significantly greater that those measured in the dead ones at $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

significantly higher compared to the living parasites when they were incubated at the highest drug concentrations (see Figs. 1 and 2). Conversely, TCBZSO concentrations obtained in dead liver flukes were twofold higher in the intact living trematode parasites compared to the dead flukes at all the concentrations assayed (Fig. 3).

FBZ was detected in the three classes of parasites at all the concentrations assayed after the ex vivo incubation. However, FBZ concentrations measured inside the nematode parasite were significantly lower than those obtained in the cestode and trematode parasites, even though the incubation time was longer (up to 90 min). While the maximum FBZ concentrations measured in living *A. suum* incubated with 10 nmol/ml KRT were slightly over 2 nmol/100 mg protein (Figs. 2 and 5), the concentrations measured in living specimens of *F. hepatica* (Fig. 6) and *M. benedeni* (Figs. 1 and 4) incubated with the same drug concentration were >20 nmol/100 mg protein. The concentrations of FBZ achieved in living *F. hepatica* (Fig. 6) were significantly higher than those reached in living *M. benedeni* (Fig. 4) (no difference with 1 nmol/ml), but this was directly correlated with the longer incubation time used for the liver fluke's assays (45 vs 15 min of incubation).

FBZ and OFZ concentrations measured inside intact living *M. benedeni* are shown in Fig. 1. FBZ and OFZ readily

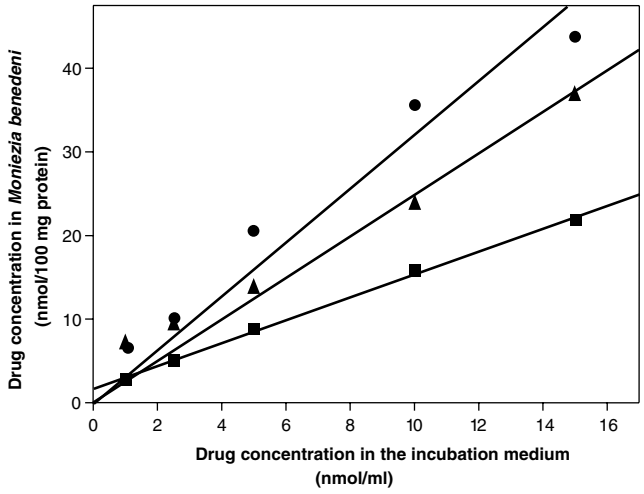

Fig. 4. Correlation between drug concentrations in the incubation medium and those measured in the cestode *Moniezia benedeni*. Linear regression analysis for: -▲- fenbendazole (FBZ) in living parasites ($r = 0.996$); -●- FBZ in dead parasites ($r = 0.989$); -■- oxfendazole (OFZ) in living parasites ($r = 0.999$).

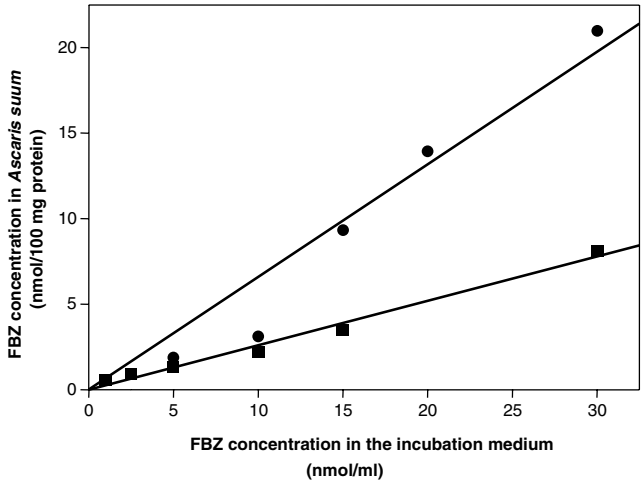

Fig. 5. Correlation (r) between fenbendazole (FBZ) concentrations measured in the incubation medium and, in -■- living ($r = 0.993$) and -●- dead ($r = 0.989$) *Ascaris suum*.

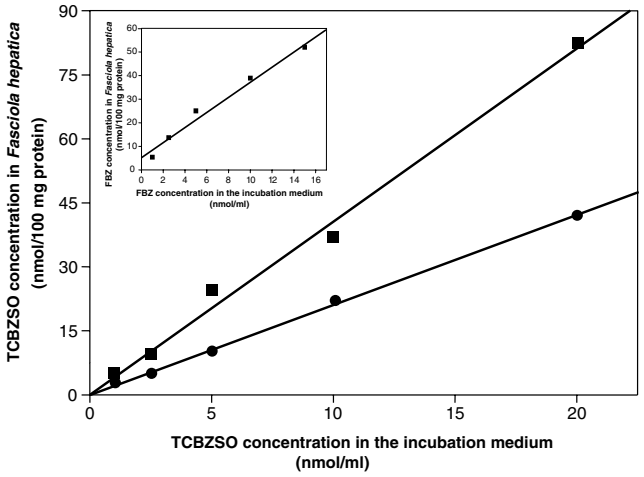

Fig. 6. Correlation (r) between triclabendazole sulphoxide (TCBZSO) concentrations measured in the incubation medium and, in -■- living ($r = 0.996$) and -●- dead ($r = 0.999$) *Fasciola hepatica*. The insert shows the correlation obtained for fenbendazole (FBZ) in living liver flukes ($r = 0.990$).

diffused into the cestode parasite after their ex vivo incubation. However, the uptake of FBZ was significantly ($p < 0.01$) greater than that observed for its sulphoxide metabolite at all the concentrations assayed.

FBZ concentrations obtained inside ligated and non-ligated liver flukes were not significantly different, 21.3 ± 1.9 vs 25.0 ± 3.5 nmol/100 mg protein, respectively. Similarly, equivalent TCBZSO concentrations were measured inside ligated and non-ligated liver flukes, indicating drug penetration into liver flukes even when the oral route had been closed off by ligation. When BSA was added to the media, TCBZSO concentrations measured inside the liver flukes were significantly lower (85%) ($p < 0.001$) compared to those amounts obtained when the parasites were incubated in the absence of the plasma protein (Fig. 7).

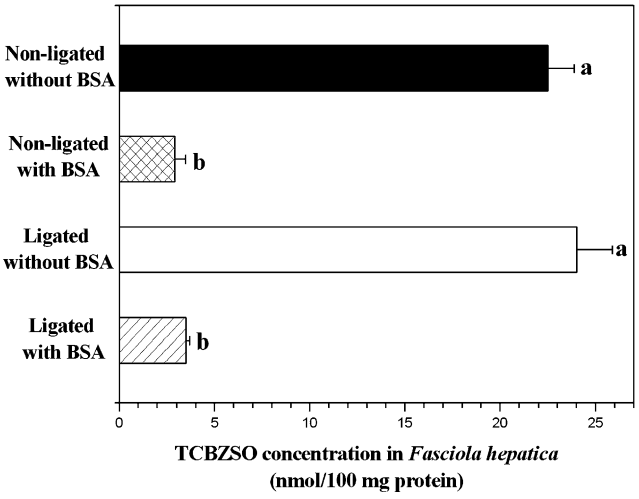

Fig. 7. Comparative ex vivo diffusion of triclabendazole sulphoxide (TCBZSO) into ligated and non-ligated *Fasciola hepatica* both in the presence and absence of bovine serum albumin (BSA). Results represent mean TCBZSO concentrations (±SD, $n = 4$) measured in the liver flukes after a 45-min incubation. Concentration values obtained for TCBZSO in non-ligated and ligated liver flukes did not reaching statistical difference. When BSA was added to the incubation medium, TCBZSO concentrations measured inside the liver flukes were significantly lower compared to those obtained when the parasites were incubated in the absence of the protein. [a,b]Different letters mean statistical difference at $p < 0.001$.

4. Discussion

The concentration gradient and time of exposure are relevant factors, among many others, in the drug absorption process. In the case of passive diffusion, as the concentration gradient increases at one side of a cell membrane, drug concentrations at the other side will increase in favour of the concentration gradient. If transport proteins are involved in the mechanism of drug absorption, saturation may follow the increase of drug concentration at one side of a membrane. Therefore, the concentration achieved at the other side does not necessary follow a linear relationship with the concentrations in the surrounding medium. For that reason, the transport mechanisms involved in drug entry into a parasite can be clarified comparing drug concentrations reached within helminth parasites incubated with different drug concentrations during a determined incubation period. This was the main goal of the present experimental work.

From the overall results reported here we can conclude that BZD anthelmintics accumulated inside parasites in favour of the concentration gradient in the three helminth parasites, *M. benedeni*, *F. hepatica*, and *A. suum*. The entry of the BZD molecules was linear over the range of concentrations assayed. These results are supported by the linear regression analyses; high correlation coefficients were obtained regardless the use of dead or living parasites (Figs. 4–6). Additionally, it is well known that lipidsolubility facilitates drug diffusion through external surface of *A. suum* (Ho et al., 1992), *M. benedeni* (Mottier et al., 2003b), and *F. hepatica* (Mottier et al., 2004b). OFZ, the sulphoxide metabolite of FBZ, is pharmacologically less active and it has a lower lipidsolubility (octanol–water partition coefficient, *Log P* 2.03) than the parent compound (*Log P* 3.93) (Mottier et al., 2003b). If the amounts of FBZ and OFZ recovered from ex vivo-incubated *M. benedeni* are compared (Fig. 1), it could be observed that the parent compound reached greater concentrations than its sulphoxide metabolite. The only way that a given drug molecule can reach a systemic tissue in a cestode parasite is by passing through its tegument, demonstrating that concentration and lipophilicity were major factors determining drug penetration. Similarly, the higher lipophilicity of albendazole (ABZ) (octanol–water partition coefficient: 3.83) (Mottier et al., 2003b) accounted for its greater penetration through the external parasite surface, compared to its sulphoxide derivative (octanol–water partition coefficient: 1.24), observed in *M. benedeni* under ex vivo conditions (Mottier et al., 2003b) and in the abomasal nematode *Haemonchus*

contortus collected from ABZ-treated infected sheep (Alvarez et al., 2000). These results are in agreement with those obtained by Alvarez et al. (2004), who suggested that physicochemical composition of the parasite's surrounding environment play a pivotal role in the process of drug access into *F. hepatica*. Therefore, the close relationship between drug concentrations in the incubation medium and those achieved in the parasite, the relevance of drug lipid-solubility and the influence of the incubation medium composition in the amount of drug that reaches the parasite, corroborate the hypothesis that BZD entry into parasites occurs by passive diffusion.

Equivalent concentrations of both TCBZSO and FBZ were measured inside ligated and non-ligated adult *F. hepatica*. These results confirm BZD entry into liver flukes even when the oral route had been closed off by ligation, which also demonstrates that trans-tegumental diffusion is a main mechanism involved in the penetration of these drugs into the liver flukes to exert their pharmacological action. Additionally, to further confirm those findings, BSA was added to the incubation medium. Since TCBZSO strongly binds to plasma proteins (≈90%, especially albumin) and *F. hepatica* is a blood-feeding trematode, the presence of albumin contributes to further elucidate the mechanism of drug entry into the fluke. The amount of TCBZSO recovered within ligated flukes incubated with albumin was 85% lower than that measured in the flukes incubated in the absence of this serum protein. The same pattern was observed in the non-ligated flukes, which demonstrated that only the small portion of unbound drug was able to diffuse into the trematode parasite. Altogether, the available information provides a strong biophysical basis for demonstrating that the main mechanism of BZD deliver into target helminth parasites is through their external surface even in a blood-sucking parasite such as the adult stages of *F. hepatica*.

Although FBZ and TCBZSO diffused into both the living and dead worms, important differences were found between them. Drug incubations with dead parasites were useful to discard possible oral ingestion of the drug and the occurrence of active drug transport, processes occurring only in living parasites/tissues. In the case of *F. hepatica*, oral ingestion of TCBZSO was also discarded with the ligature experiment. In a similar experiment with *A. suum*, Ho et al. (1992) did not observe differences in uptake kinetics of permeants (hydrocortisone, *p*-nitrophenol, and urea) between ligated and non-ligated nematodes. FBZ concentrations recovered from the tissues of the dead cestodes/nematodes over time were significantly greater (at the highest drug concentrations assayed) compared to the living parasites. These differences in drug uptake may be related to the morphological/functional properties of the parasite's external surfaces. Traditionally, the nematode's cuticle has been considered to be a barrier limiting entry of larger molecules into the parasite. The cuticle consists of (1) collagen like proteins that form the medial and basal layers, (2) non-collagen proteins that form the epicuticular and external cortical regions and, (3) non-structural proteins associated with the external surface (Fetterer and Rhoads, 1993). The water-filled, porous, negatively charged collagenous matrix of *A. suum* permits the passage of molecules by molecular size restricted diffusion within an electrostatic field of force. If the molecule is sufficiently small, it could traverse the aqueous-filled negatively charged collagen matrix of the cuticle (Ho et al., 1992). The external surface of *A. suum* can be breached by drugs and the rate-determining barrier for passive transport is the lipoidal hypocuticle tissue, in which the rate-determining factors are the intrinsic lipid–water partition coefficient, pH/pK_a relationship, molecular size and restriction by the pores influencing the diffusion of uncharged molecules, indicating that the transcuticular transport of weak acids and bases will be controlled largely by the pH at this surface, since, in the absence of facilitated transport, only unionized species can partition across a lipoidal surface (Sims et al., 1992). This nematode parasite excretes a number of volatile fatty acids as well as lower levels of two nonvolatile organic acids (end products of carbohydrate metabolism) via the transcuticular route at sufficient rates to establish and maintain a buffered microenvironmental pH of approximately 5.0 in the aqueous space of the pores of the cuticle (Sims et al., 1992). It has been also proposed for cestode parasites that organic acid end-products of carbohydrate metabolism are excreted via the transtegumental pathway, forming an acidic microenvironment in the immediate vicinity of the tegument (Halton, 2004; Uglem, 1991). The results shown here support previously available data (Sims et al., 1992) in that the acidic pH values at the parasite's external surface would influence the transport properties of weak acidic and basic molecules which should be considered in the design of delivery systems for anthelmintic drugs. Most drugs are weak organic bases and exist in solution as both non-ionised and ionized forms (Baggot, 1977). While the poor lipid-solubility of ionized molecules excludes them from passive diffusion, lipophilic, non-ionised moieties passively diffuse across cell membranes until equilibrium is established. FBZ is a weak base (p*K*a = 7–8, Lanusse and Prichard, 1993) that may largely exists as its ionized form in the acidic environment of the nematode/cestode surface, which helps to explain its limited diffusion across the cuticle/tegument in the intact living parasites. The lack of this barrier in the dead worms permitted a greater deliver of the BZD molecules into *A. suum* and *M. benedeni*, such as was shown here. The results obtained in the current experimental work comparing living and dead worms demonstrated how trans-cuticular/tegumental absorption of weak bases, such as BZD compounds, may be affected by the excretion of organic acid metabolites in living parasites. The argument above may explain the greater concentrations found in the dead worms due to a lack of the organic acids barrier. Furthermore, these results are supported by the fact that oral ingestion and tegumental/cuticular damage were ruled out by the complementary experimental work reported here.

Contrarily to the results obtained for nematodes and cestodes, TCBZSO concentrations measured inside the living trematode parasite were twice compared to those

obtained in the dead flukes at all the concentrations assayed. According to all the experimental evidence shown in the current work, the incubations with dead *F. hepatica* showed that a viable parasite is needed to concentrate BZD anthelmintics, although a significant percentage of the drug (50%) is still bound to tissue components of the dead parasite. These results are in accordance with those obtained by Bennett and Köhler (1987) and could be related to an active process, where the concentrations assayed resulted insufficient to saturate the implicated uptake mechanism. Therefore, the observed uptake behaviour of the drug was similar to that occurring when a passive diffusion mechanism (first order kinetics) is involved. The drug concentration range used in the current ex vivo experiments are in agreement with those measured in bile after in vivo treatments in sheep (Hennessy et al., 1987, 1993a,b), which agrees with the experimental evidences showing that drug concentrations achieved within a target parasite are close related to the drug levels achieved at the site of parasite location (Alvarez et al., 1999, 2000), regardless the characteristics of the implicated drug entry mechanism.

The complex structure of the cuticle compared with the tegument could explain the differences observed between the nematode and trematode/cestode parasites in the current trials, where it was clearly shown that FBZ diffusion in *A. suum* was markedly lower than those measured in the trematode *F. hepatica* and the cestode *M. benedeni*. Despite the structural differences between cuticle and tegument, the mechanism of drug entry to both types of structures seems to be equally dependent on lipophilicity as a major physicochemical determinant of drug capability to reach therapeutic concentrations within the target parasite.

In conclusion, since the concentration gradient, drug lipidsolubility, physico-chemical characteristics of the incubation medium and the structure and composition of the external surface of helminth parasites are critical for the penetration of BZD molecules through helminth external surfaces, it is clear that passive diffusion is the main mechanism implicated in the entry of these anthelmintic drugs into cestodes, trematodes and nematodes. It is also likely that the same phenomenon may be involved for other high lipophilic anthelmintic drugs. However, this requires additional experimental work and it is currently under investigation.

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