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# Comparative assessment of albendazole and triclabendazole ovicidal activity on *Fasciola hepatica* eggs

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ABSTRACT

The benzimidazole compounds albendazole (ABZ) and triclabendazole (TCBZ) are both effective against *Fasciola hepatica*, although ABZ is only effective against adult flukes. Additionally, ABZ is a broad-spectrum nematodicidal compound with well-known ovicidal activity. However, no data on the ovicidal effect of TCBZ against *F. hepatica* eggs are available. The work reported here evaluated the comparative ovicidal effect of ABZ, TCBZ and their sulphoxide metabolites on *F. hepatica* eggs recovered from bile of sheep artificially infected with either a TCBZ-susceptible (Cullompton) or a TCBZ-resistant (Sligo) isolate of *F. hepatica*. Additionally, the effects of different non-flukicidal methylcarbamate benzimidazole compounds on the hatching of *F. hepatica* eggs were evaluated. Eggs (500 eggs/mL, $n = 4$) were incubated for 12 h either with TCBZ, TCBZ sulphoxide (TCBZ.SO), ABZ (5, 10 and 20 nmol/mL) or without drug (untreated control) (Experiment 1). Additionally, the effect of TCBZ and TCBZ.SO (5 nmol/mL) on egg hatchability was examined after a long (15 days) drug exposure (Experiment 2). Furthermore, the ovicidal effect of ABZ and ABZ.SO at different concentrations (5, 1, 0.5, 0.1and 0.05 nmol/mL) (Experiment 3), and the effect of fenbendazole (FBZ), oxfendazole (OFZ), mebendazole (MBZ), flubendazole (FLBZ) (5 nmol/mL) and reduced-FLBZ (R-FLBZ) (2 μg/mL) on fluke eggs, were evaluated after a 12-h exposure (Experiment 4). Egg hatch was assessed by direct microscopic observation after incubation at 25 °C for 15 days. TCBZ and TCBZ.SO did not affect egg hatch after a 12-h incubation. A similar result was obtained after a much longer drug exposure (15 days) (Experiment 1 and 2). However, a significant ($P < 0.05$) inhibition of egg hatch was observed in ABZ- and ABZ.SO-incubated eggs (Experiments 1 and 3). Additionally, the non-flukicidal compounds (Experiment 4) affected egg hatchability, particularly FLBZ and R-FLBZ. In conclusion, ABZ and ABZ.SO had a clear inhibitory effect on egg development of *F. hepatica*. However, the most extensively used flukicidal compound, TCBZ, and its main sulphoxide metabolite, did not affect egg hatch, even in TCBZ-susceptible flukes.



## 1. Introduction

Fascioliasis, caused by the trematode liver fluke *Fasciola hepatica*, is the cause of considerable loss in sheep and cattle production systems all over the world (Roberson and Courtney, 1995). Benzimidazoles (BZD) are broad-spectrum anthelmintic compounds widely used in human and

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veterinary medicine to control nematode, cestode and trematode infections (McKellar and Scott, 1990). The BZD compounds currently marketed as anthelmintics can be grouped as BZD thiazolyls, BZD methylcarbamates, pro-BZD and halogenated BZD thiols (Lanusse and Prichard, 1993). Only a few molecules within the BZD chemical family demonstrate activity against *F. hepatica* (Fairweather and Boray, 2005). The halogenated derivative triclabendazole (TCBZ) is the most effective because of its excellent activity against adult and juvenile flukes (Boray et al., 1983). Consequently, it is the most widely used and this has led to the selection and emergence of TCBZ-resistant fluke populations in several areas of the world (data reviewed by Fairweather, 2005, 2009). TCBZ is structurally quite different from other BZD, having neither a carbamate nor a thiazolyl ring in the carbon 2 position (Campbell, 1990), but having three chloride atoms in its molecule. Albendazole (ABZ) is the only BZD methylcarbamate recommended for the control of fascioliasis in domestic animals, despite its activity being restricted to flukes older than 12 weeks (McKellar and Scott, 1990). Fenbendazole (FBZ), a similar BZD methylcarbamate widely used in veterinary medicine as a nematodicidal drug, is not as effective as ABZ against *F. hepatica*, but a single treatment of 5 mg/kg reduced *F. gigantica* infection in sheep by up to 95% (Roberson and Courtney, 1995).

The BZD anthelmintics are extensively metabolised in all mammalian species studied (Gottschall et al., 1990; Lanusse and Prichard, 1993). TCBZ parent drug is not detected in plasma after its oral administration to sheep, indicating that it is completely removed from portal blood by the liver following absorption (Hennessy et al., 1987). TCBZ is oxidised to form the metabolites triclabendazole sulphoxide (TCBZ.SO) and triclabendazole sulphone (TCBZ.$SO_2$). Since TCBZ.SO is the major metabolite found in the plasma of TCBZ-treated sheep, and only low concentrations of the parent compound are detected in bile (unpublished observations), TCBZ.SO has been postulated to be responsible for the activity against liver flukes (Fairweather, 2005, 2009). Similar to that observed for TCBZ, ABZ is not found in the bloodstream after enteral administration to sheep (Marriner and Bogan, 1980) and cattle (Prichard et al., 1985). ABZ oxidations lead to more polar and less anthelmintically active metabolites. In terms of binding to parasite tubulin, the ABZ parent drug is more potent than its sulphoxide metabolite (ABZ.SO), while the sulphone (ABZ.$SO_2$) is an inactive derivative (Lacey, 1990; Lubega and Prichard, 1991).

In addition to their excellent nematodicidal efficacy, BZD anthelmintics demonstrate ovicidal activity, as eggs of many nematode species fail to hatch after BZD exposure in the gut contents (Lacey, 1988). Additionally, some BZD methylcarbamate compounds have activity against *F. hepatica* eggs (Coles and Briscoe, 1978). However, as far as we know, no data on the ovicidal effect of TCBZ against *F. hepatica* eggs is available. The main goals of the current trial were: (a) to evaluate the comparative ovicidal effect of TCBZ, ABZ and their respective sulphoxide metabolites on *Fasciola hepatica* eggs obtained from TCBZ-susceptible and -resistant isolates; and (b) to assess the effects of different non-flukicidal methylcarbamate benzimidazole compounds on the hatching of *F. hepatica* eggs.

## 2. Material and methods

### 2.1. Chemicals

Pure reference standards of TCBZ and TCBZ.SO (Novartis Animal Health, Basel, Switzerland), ABZ FBZ, OFZ, MBZ (Schering Plough, Kenilworth, USA), FLBZ and its reduced metabolite (R-FLBZ) (Janssen Animal Health, Beerse, Belgium) were used for the experimental assays. The solvents (methanol or DMSO) used for drug dissolution were of analytical grade (Anedra, Buenos Aires, Argentina).

### 2.2. Collection of F. hepatica eggs

Six (6) Corriedale sheep were orally infected with 200 metacercariae of *F. hepatica* contained in a gelatine capsule. Four (4) animals were infected with a TCBZ-susceptible isolate (named Cullompton) and the other two (2) sheep with a TCBZ-resistant isolate (named Sligo). For details of the history of the two isolates, see Robinson et al. (2004) and McConville et al. (2009). Sixteen (16) 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 (AVMA, 2001). *F. hepatica* eggs (both isolates) were directly recovered from the bile of each infected sheep. After several washes with tap water, eggs were suspended in water (500 eggs/mL).

### 2.3. Experimental design

Four separate studies (Experiments 1–4) were performed, as follows:

*Experiment 1*: It was performed to evaluate the comparative ovicidal effect of the fukicidal compounds TCBZ, TCBZ.SO and ABZ on *Fasciola hepatica* eggs obtained from a TCBZ-susceptible or a TCBZ-resistant *F. hepatica* isolate. Fluke eggs (500/mL, $n = 4$) from both isolates (TCBZ-susceptible or -resistant) were incubated (25 °C) for a 12-h period with either TCBZ, TCBZ.SO or ABZ at a final concentration of either 5, 10 or 20 nmol/mL. They are pharmacologically relevant concentrations obtained from previous studies where the bile concentrations of these BZD compounds were measured after conventional treatments in sheep (Hennessy et al., 1987; Alvarez et al., 2000). A 12-h period of drug–egg contact represents the approximate time of egg exposure to the drug after an *in vivo* treatment. Untreated eggs were incubated as control assays. Untreated and treated eggs were gently washed (3×) to facilitate drug removal, and kept in darkness at 25 °C for 15 days. After this period, the trematode eggs were exposed to daylight for 1 h. When this time had elapsed, 1 mL of 10% (v/v) buffered formalin was added to each tube in order to stop egg hatching. Hatched and unhatched eggs were evaluated using an optical microscope (40× magnification). Approximately 80 eggs were counted to estimate the proportion of hatched eggs in each tube.

*Experiment 2*: Based on the results obtained from Experiment 1, the potential ovicidal effect of TCBZ and TCBZ.SO was determined after 15 days of incubation (long-lasting exposure), without drug removal. *F. hepatica* eggs (500/mL, $n = 4$) collected from sheep infected with the TCBZ-susceptible isolate were incubated at 25 °C with either TCBZ or TCBZ.SO (5 nmol/mL) for 15 days (without drug removal). Untreated eggs were incubated as the control. Untreated and treated eggs were kept in darkness at 25 °C for 15 days and then processed as described for Experiment 1.

*Experiment 3*: It was performed to assess the comparative ovicidal effect of different concentrations of ABZ and its sulphoxide metabolite on *F. hepatica* eggs obtained from a TCBZ-susceptible isolate of *F. hepatica F. hepatica* eggs (500/mL, $n = 4$) collected from sheep infected with the TCBZ-susceptible isolate were incubated at 25 °C with either ABZ or ABZ.SO at 5, 1, 0.5, 0.1 or 0.05 nmol/mL for a 12-h period and then processed as described for Experiment 1.

*Experiment 4*: The comparative ovicidal effect of several non-flukicidal methyl carbamate compounds was evaluated. *F. hepatica* eggs (500/mL, $n = 4$) collected from sheep infected with the TCBZ-susceptible isolate were incubated at 25 °C with either FBZ, OFZ, MBZ, FLBZ (5 nmol/mL) or R-FLBZ (2 μg/mL) for a 12-h period and then processed as described for Experiment 1. The reduction percentage of *F. hepatica* egg hatching induced by non-flukicidal BZD compounds was estimated using the following formula:

$$\text{hatch reduction} = \frac{\%\ \text{of hatched eggs after drug incubation}}{\%\ \text{of hatched eggs in the control}} \times 100$$

### 2.4. Statistical analysis of the data

The percentages of egg hatch are reported as the arithmetic mean ± standard deviation (SD). Parametric ANOVA + Tuckey's tests were used for the statistical comparison of the egg hatch data obtained from each experiment. A value of $P < 0.05$ was considered statistically significant. The statistical analysis was performed using the Instat 3.0 Software (Graph Pad Software, CA, USA).

## 3. Results

The mean egg hatch percentage obtained for untreated eggs in the different experiments ranged between 45.2 and 79.8% (TCBZ-susceptible isolate) and from 39.4 to 50.2% (TCBZ-resistant isolate). The percentages (mean ± SD) of hatched TCBZ-susceptible and -resistant *F. hepatica* eggs after incubation with TCBZ, TCBZ.SO and ABZ for a 12-h period (Experiment 1) are compared in Fig. 1. Incubation of eggs of both isolates in TCBZ or TCBZ.SO did not lead to a significant reduction in egg hatch, at any of the concentrations tested. Conversely, ABZ induced a significant ($P < 0.05$) hatch reduction in eggs obtained from both TCBZ-susceptible and -resistant flukes.

The hatching of TCBZ-susceptible *F. hepatica* eggs after incubation with either TCBZ or TCBZ.SO for a 15-day period at 25 °C (Experiment 2) are summarized in Table 1. Clearly, even after a long-lasting exposure (15 days), both TCBZ and TCBZ.SO failed to inhibit egg development. In fact, similar egg hatch values were observed in control (60.0 ± 18.4%), TCBZ-treated (71.0 ± 20.4%) and TCBZ.SO-treated (64.8 ± 6.40%) eggs.

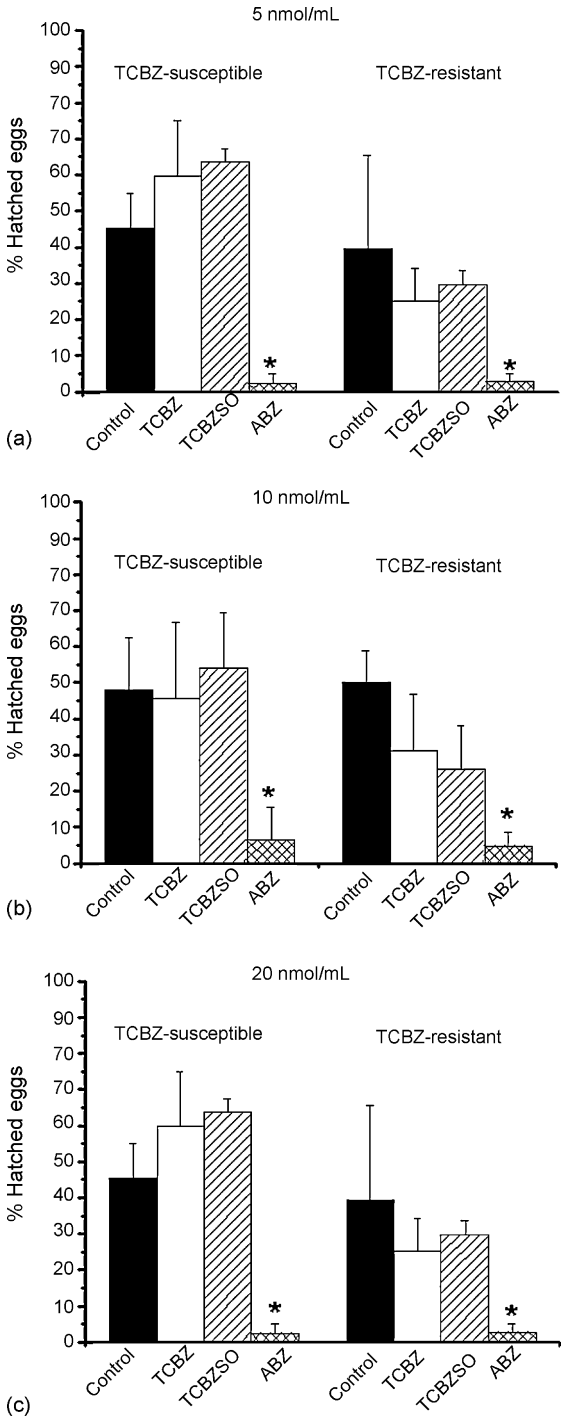


**Fig. 1.** Percentage (mean ± SD) of hatched eggs from TCBZ-susceptible and -resistant *F. hepatica* isolates after their incubation with triclabendazole (TCBZ), triclabendazole sulphoxide (TCBZ.SO) and albendazole (ABZ) for a 12-h period at the concentrations of 5 nmol/mL (a), 10 nmol/mL (b) and 20 nmol/mL (c). *Indicates statistically significant differences ($P < 0.05$).

**Table 1**
Hatching of eggs from a triclabendazole-susceptible *F. hepatica* isolate after incubation with either triclabendazole (TCBZ) or TCBZ sulphoxide (TCBZ.SO) for a 15-day period (long-lasted incubation without drug removal) at 25 °C. Values are expressed as mean ± SD.

| Drug concentration nmol/mL ($n = 4$) | Egg hatch percentage | | |
|---|---|---|---|
| | Control | TCBZ | TCBZ.SO |
| 5 | 60.0 ± 18.4 | 71.0 ± 20.4 | 64.8 ± 6.40 |

Values are not statistically significant ($P > 0.05$).

**Table 2**
Hatching of eggs from a triclabendazole-susceptible *F. hepatica* isolate after incubation with either albendazole (ABZ) or ABZ sulphoxide (ABZ.SO) at different drug concentrations for a 15-day period (with drug removal after a 12 h incubation) at 25 °C. Values are expressed as mean ± SD.

| Drug concentration nmol/mL ($n = 4$) | Egg hatch percentage | |
|---|---|---|
| | ABZ | ABZ.SO |
| 5 | $3.7 \pm 4.2^{a}$ | $4.6 \pm 3.0^{a}$ |
| 1 | $6.0 \pm 4.9^{a}$ | $4.8 \pm 4.2^{a}$ |
| 0.5 | $2.7 \pm 2.4^{a}$ | $20.2 \pm 7.5^{c}$ |
| 0.1 | $11.6 \pm 3.8^{a}$ | $41.4 \pm 7.6^{d}$ |
| 0.05 | $13.2 \pm 6.2^{a}$ | $58.4 \pm 10.7^{b}$ |
| 0 (control) | $72.6 \pm 4.1^{b}$ | $72.6 \pm 4.1^{b}$ |

Values with different superscripts are statistically different ($P < 0.05$).

The percentages (mean ± SD) of hatched TCBZ-susceptible *F. hepatica* eggs after incubation with either ABZ or ABZ.SO for a 12-h period (Experiment 3) are shown in Table 2. While ABZ significantly reduced egg hatch at a concentration as low as 0.05 nmol/mL ($P < 0.05$), an ovicidal effect of ABZ.SO was not observed at this concentration. Furthermore, at higher concentrations (0.1 and 0.5 nmol/mL), ABZ.SO egg hatch reduction was lower than that observed for the ABZ parent drug.

Fig. 2 shows the hatching reduction (mean ± SD) in fluke eggs induced by the non-flukicidal benzimidazole compounds FBZ, OFZ, MBZ, FLBZ and R-FLBZ (Experiment 4). All the assayed compounds affected the egg hatch (significant differences compared with the untreated control). Additionally, the egg hatch reduction induced by FLBZ and its reduced metabolite (R-FLBZ) were significantly ($P < 0.05$) higher than those observed for OFZ and MBZ.

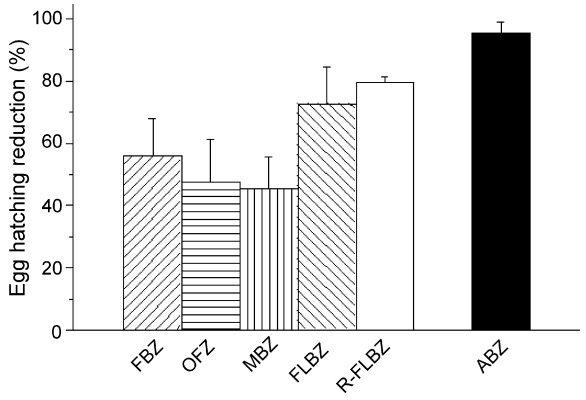


**Fig. 2.** Reduction (mean ± SD) in hatching of *F. hepatica* eggs induced by nematodicidal benzimidazole compounds fenbendazole (FBZ), oxfendazole (OFZ), mebendazole (MBZ), flubendazole (FLBZ) and its reduced flubendazole metabolite (R-FLBZ). Data on albendazole (ABZ) reduction are from Experiment 1, and are shown here only for comparative purpose.

## 4. Discussion

The main goal of the current work was to evaluate the potential of the main available flukicidal drug TCBZ and its sulphoxide metabolite to inhibit the hatching of *F. hepatica* eggs. TCBZ and TCBZ.SO did not demonstrate any ovicidal effect on eggs recovered from both TCBZ-susceptible and -resistant *F. hepatica* isolates. In contrast, ABZ inhibited egg development in both *F. hepatica* isolates assayed. Both kinetic and/or dynamic factors may help to explain the lack of effect on egg hatching observed for TCBZ and its sulphoxide metabolite.

The effect of some BZD anthelmintics on nematode eggs has been well characterized. Nematode egg viability was reduced up to 0.1% 12 h after fenbendazole treatment in calves (Miller and Morrison, 1992). An egg hatch test is recommended to detect anthelmintic resistance to BZD compounds in nematodes (Coles et al., 2006). The ovicidal action of BZD methylcarbamates in nematodes is related not only to the drug affinity for β-tubulin, but also to its ability to penetrate the egg shell and accumulate within the egg. It has been suggested that egg hatching inhibition depends on the drug's hydrophobic nature, where increased activity correlates with higher fat solubility (Lacey, 1988). While highly hydrophilic drugs fail to penetrate the egg shell, highly lipophilic drugs bind to the shell components and may fail to concentrate within the egg, where the target of TCBZ action is present. Furthermore, TCBZ has a high affinity for different proteins, which may also account for the "sequestration" of TCBZ and/or its metabolites within the eggshell. The octanol–water partition coefficient (*log P*) is used as an indicator of drug lipid solubility. Previous studies have demonstrated that optimal ovicidal activity (in nematodes) can only be achieved over a relatively narrow "window" of *log P* values, from 1 to 3.4 (Lacey, 1988). Compounds with *log P* values below or above this range do not have ovicidal activity in nematode eggs. The *log P* values for TCBZ and TCBZ.SO have been established to be 3.49 and 3.62, respectively (Mottier et al., 2004). Regardless of the differences in structure between nematode and trematode eggs, if the "window" proposed for nematode eggs is applicable to trematode eggs, the lack of ovicidal activity of TCBZ/TCBZ.SO may be explained due to its difficulty in concentrating within the egg. However, since the lack of ovicidal activity is observed even when the eggs are exposed to TCBZ/TCBZ.SO for 15 days, or when relatively high concentrations are used, it is likely that the lack of effects on egg hatch may not only be associated with a limitation of their distribution pattern within the egg structure, but also with a different TCBZ mode of action.

The mode of flukicidal action of TCBZ has not yet been established. However, the primary mode of action of BZD anthelmintics in egg hatching involves inhibition of microtubule-dependent processes (Lacey, 1988). Since TCBZ is a BZD compound, a disruption of the microtubule-based processes as a result of binding to the β-tubulin molecule may be expected (Fairweather, 2005,

2009). In fact, several morphological and immunocytochemical data support a β-tubulin-mediated TCBZ action (Stitt and Fairweather, 1992, 1993, 1994, 1996; McConville et al., 2006). BZD compounds bind to the colchicine binding site on the β-tubulin molecule in nematodes (Lacey, 1988). *F. hepatica* is more refractive to colchicine action (Stitt and Fairweather, 1992) and, as a consequence, TCBZ may target an alternative binding site on the tubulin molecule. In fact, differences in a number of amino acids of the primary sequence of *F. hepatica* β-tubulin have been described (Robinson et al., 2002; Ryan et al., 2008). However, if TCBZ action is associated with microtubule depolymerisation, some egg damage would be expected considering that the drug may accumulate within the egg in therapeutic concentrations. Since TCBZ and TCBZ.SO do not have any effect on egg hatch, it is likely that other non-microtubule related mechanism of action may be implicated in the fasciolicidal activity of TCBZ.

In contrast to the lack of activity of TCBZ/TCBZ.SO against egg hatch reported here, TCBZ.SO has been shown to affect eggs produced by drug-exposed flukes, decreasing egg production and affecting reproductive structures (testis and vitelline follicles) under *ex vivo*/*in vitro* conditions (Shaw et al., 2007). Similarly, closantel produced a decrease in fluke egg production (attributed to its inhibitory effects on feeding and intermediary metabolism), but did not affect egg hatching (Hanna et al., 2006).

ABZ showed excellent ovicidal activity against *F. hepatica* eggs. Interestingly, this effect was observed in eggs recovered from both TCBZ-susceptible and -resistant *F. hepatica* and agrees with some clinical efficacy results which demonstrated that ABZ is active against mature adult TCBZ-resistant *F. hepatica* (Coles and Stafford, 2001). The ovicidal effect was also observed for ABZ.SO in the present study. However, while ABZ was active at concentrations as low as 0.05 nmol/mL, the highest ovicidal activity for the sulphoxide metabolite was observed at 1 or 5 nmol/mL. The affinity of ABZ.SO for parasite β-tubulin is lower to that observed for the *sulphide*/parent ABZ compound (Lacey et al., 1987; Lubega and Prichard, 1991); as a consequence, ABZ.SO has lower pharmacological activity than ABZ. In fact, ABZ demonstrated greater potency than ABZ.SO in nematode motility *ex vivo* studies (Petersen et al., 1997). On the other hand, the higher lipophilicity of ABZ compared to its sulphoxide metabolite (Mottier et al., 2003) may facilitate its greater penetration into the fluke eggs. Higher accumulations of ABZ were observed in specimens of *F. hepatica* and *Ascaris suum* (Alvarez et al., 2001) and in *Moniezia expansa* (Alvarez et al., 1999) compared to that observed for the more polar ABZ.SO metabolite after *ex vivo* incubations.

FBZ, OFZ, MBZ and FLBZ are indicated mainly for the control of adult and larval stages of gastrointestinal nematodes in several domestic species, including cattle, sheep, goats, pigs, horses, dogs, cats, chickens, turkeys and game birds. None of them are indicated against *F. hepatica* (Fairweather and Boray, 2005). However, some ovicidal activity against *F. hepatica* eggs was observed for these compounds. In particular, FLBZ demonstrated a marked effect on egg hatching, compared to other methylcarbamate compounds. In fact, the potential flukicidal activity of FLBZ has not yet been investigated, and probably this compound has some pharmacological activity against adult liver flukes. Interestingly, the R-FLBZ metabolite showed a reduction of egg hatch of almost 80%, which is the first description of some kind of pharmacological activity reported for this metabolite. The relevance of this finding is supported by the fact that R-FLBZ is the main FLBZ metabolite found in plasma of treated sheep (Moreno et al., 2004). Besides, this metabolite has demonstrated equivalent ability to accumulate (under *ex vivo* conditions) into target parasites to that observed for the parent FLBZ compound (Moreno et al., 2004).

In conclusion, ABZ and ABZ.SO had a clear inhibitory effect on *F. hepatica* egg development. However, the most extensively used flukicidal compound, TCBZ, and its main sulphoxide metabolite did not affect the egg hatch, even in TCBZ-susceptible flukes. These findings complement previous work on the understanding of the comparative flukicidal activity of TCBZ and ABZ.

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