Identification of a field isolate of *Fasciola hepatica* resistant to albendazole and susceptible to triclabendazole

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Running title: *Fasciola hepatica isolate resistant to albendazole*

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Abstract

The experiments described here were designed to characterize the status of susceptibility/resistance to albendazole (ABZ) and triclabendazole (TCBZ) of a *Fasciola hepatica* isolate (named CEDIVE isolate) recovered from infected sheep from in Gualeguay (Argentina) and maintained under laboratory conditions. Two separate clinical efficacy experiments were performed. Experiment 1: Sheep artificially infected with the CEDIVE isolate were randomly distributed into an untreated control and an ABZ (7.5 mg/kg) treated groups (n= 4 each). The systemic exposure of ABZ metabolites was assessed in those ABZ-treated infected animals. Additionally, an untreated control group and a TCBZ (10 mg/kg) treated group was included in Experiment 2 (n=4 each). The fluckicidal efficacy of ABZ and TCBZ was assessed by comparison of the number of flukes recovered from untreated and treated sheep at 15 days post-treatment. The efficacy against the CEDIVE isolate of *F. hepatica* was 29% (ABZ) and 100 % (TCBZ). The plasma drug exposure (expressed as AUC and Cmax) observed in the ABZ treated animals (Experiment 1), was in agreement with data obtained in previous studies, which indicate that the low ABZ efficacy was not related to the quality of the pharmaceutical product and/or to a low systemic availability of the active drug/metabolite. The results reported here, clearly show that the CEDIVE isolate of *F. hepatica* behaves as resistant to ABZ and susceptible to TCBZ.

Key words: *Fasciola hepatica* isolate, resistance, albendazole, triclabendazole.

Introduction

The trematode *Fasciola hepatica* is a cosmopolitan parasite which causes considerable loss in sheep and cattle production systems all over the world (Boray, 1994). As a result of climate change, there has been a dramatic resurgence of sheep and cattle fascioliasis in different regions of the world (Mitchell, 2002). Chemotherapy is the available main tool to control liver flukes. However, the frequent use of effective fluckicidal compounds leads to the development of resistance. Although resistance in flukes has not yet reached the levels roported in nematodes (Wolstenholme et al., 2004), failures of rafoxanide and closantel have been reported (Fairweather and Boray, 1999). Most of the reports involving drug resistance in *F. hepatica* are related to triclabendazole (TCBZ), the most used flukicidal drug in veterinary medicine (Coles and Stafford, 2001). Unlike other benzimidazole (BZD) compounds, the halogenated derivative TCBZ has been shown to have an excellent efficacy against the mature and immature stages of *F. hepatica* (Boray et al., 1983). However, TCBZ activity appears to be restricted to the liver fluke and the lung fluke, *Paragonimus* spp. (Weber, et al., 1988; Calvopina et al., 1998), since the drug is inactive against nematodes, cestodes and other trematode parasites (*Dicrocoelium dendriticum*, *Paramphistomun* spp. and *Schistosoma mansoni*). The intensive use of TCBZ in endemic areas of fascioliasis has resulted in the development of liver flukes resistant to this compound (Overend and Bowen, 1995; Mitchell, et al., 1998; Moll et al., 2000; Thomas et al., 2000; Olaechea et al., 2011), which is considered a major problem for veterinary therapeutics.

Albendazole (ABZ) is the only BZD methylcarbamate recommended to control fascioliasis in domestic animals, despite its activity is restricted to flukes older than 12 weeks (McKellar and Scott, 1990). A previously reported work (Coles and Stafford,

2001) has shown that ABZ was active against a TCBZ-resistant isolate of *F. hepatica*. On the other hand, resistance to both, ABZ and TCBZ in *F. hepatica*, has been reported in Spain (Alvarez-Sanchez et al., 2006). Since no alternative methods for fluke control are available, it is critical to understand how resistance is developed in order to limit its impact on livestock production (Wolstenholme et al., 2004). However, there are relatively few clearly defined isolates of *F. hepatica* available for study, and when new isolates become available they should be subjected to careful scrutiny to establish their response to different anthelmintic drugs (Fairweather, 2011). In order to gain a deeper insight on the mechanism involved on drug resistance in *F. hepatica*, it is relevant to investigate this resistance mechanisms in well characterized fluke isolates. The current work was designed to characterize the status of susceptibility/resistance to ABZ and TCBZ for a field isolate of *F. hepatica*, recovered from an Argentinean farm, which was initially suspected to be susceptible to TCBZ, maintained and artificially produced under laboratory conditions.

Material and Methods

Chemicals

Pure (≥99%) standards of ABZ, ABZ-sulphoxide (ABZSO), ABZ-sulphone ($ABZSO_2$), and oxibendazole (OBZ) used as internal standard (IS), were use in the present experiment. The commercial formulation of ABZ (Baxen 3.8%®, suspension) was from Tecnofarm, Argentina. TCBZ formulation (Fasinex® 10%, suspension) was from Novartis, Argentina. All the solvents (acetonitrile and methanol) used during the extraction and drug analysis were HPLC grade and purchased from Baker Inc. (Phillipsburg, NJ, USA). Water was double distilled and deionized using a water

purification system (Simplicity®, Millipore, Brazil). Buffer salts ($ClNH_4$) were purchased from Baker Inc. (Phillipsburg, NJ, USA).

Experimental design

Animals

Sixteen (16) healthy male Corriedale sheep (48.6 ± 2.6 kg) aged 24-26 months were involved in this trial. Animals were housed during the experiment and for 15 days before the start of the study. Animals were fed on a commercial balanced concentrate diet. Water was provided *ad libitum*. Animal procedures and management protocols were carried out in accordance with the 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 internationally accepted animal welfare guidelines (AVMA, 2001).

F. hepatica isolate

The *F. hepatica* isolate characterized in the current work (named CEDIVE isolate) was recovered from the farm "San Julian", located in the Department of Gualeguay, Entre Ríos, Argentina (32º 52´ S; 59º 25´ O). The farm, with a size of 1200 hectareas, is dedicated to raising cattle (700 heads) and sheep (1000 heads). In this farm, 4 to 5 anthelmintic treatments per year have been used by several years in sheep. The treatments were based in the use of BZD compounds (mainly ABZ and oxfendazole), ivermectin and closantel. All anthelmintic treatments used in sheep were directed against gastrointestinal nematodes (*Haemonchus* spp and *Trichostrongylus* spp.). Although the presence of liver flukes has been found in both cattle and sheep, no specific treatments

against *F. hepatica* has been implemented, were it not for sporadic drenches in outbreaks, which mainly involved triclabendazole or closantel. Eggs of the *F. hepatica* isolate were recovered from the bile ducts of two sacrificed sheep, and subsequently maintained in donor animals and snails under laboratory conditions at the "Centro de Diagnóstico e Investigaciones Veterinarias" (CEDIVE), Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata, Chascomús, Argentina.

Experimental trials

The drug-susceptibility characterization of the CEDIVE isolate of *F. hepatica* involved two separate experiments. ***Experiment 1:*** Each animal was orally infected with two hundred (200) metacercariae of the *F. hepatica* CEDIVE isolate. Sixteen weeks after infection, animals were randomly distributed into two experimental groups (n= 4 each): Control Group, which represented the untreated control and the ABZ Group, where the animals were treated with ABZ (Baxen 3.8%®, Tecnofarm, Argentina) by the i.r. route at the dose of 7.5 mg/kg. The ABZ reference product (Valbazen, Pfizer) was discontinued in Argentina. Since there were not available data on the plasma exposure of ABZ/metabolites after the use of the Baxen 3.8% formulation and, in order to discard treatment failures derived from the use of a poor quality formulation, a plasma pharmacokinetic study was also performed. For the pharmacokinetic study blood samples were taken by jugular venipunctures into heparinized Vacutainers® tubes (Becton Dickinson, USA) before administration (time 0) and at 1, 3, 6, 9, 12, 15, 24, 28, 32, 48 and 54 h post-treatment. Plasma was separated by centrifugation at 3000 ***g*** for 15 min, placed into plastic tubes and frozen at -20 ºC until analysis by high performance liquid chromatography (HPLC).

Experiment 2: Each experimental animal was orally infected with two hundred (200) metacercariae of the *F. hepatica* CEDIVE isolate. Sixteen weeks after infection, animals were randomly distributed into two experimental groups (n= 4 each): Control Group, which represented the untreated control and TCBZ Group, in which animals were treated with TCBZ (Fasinex®, Novartis, Argentina) by the i.r. route at the dose of 10 mg/kg.

Clinical efficacy study

Fifteen (15) days after treatment all animals were stunned and exsanguinated immediately. Adult *F. hepatica* specimens were recovered from the main bile ducts and the gall bladder of each sheep and counted according to the World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P) guidelines (Wood et al., 1995). The efficacy of each anthelmintic treatment was determined by the comparison of *F. hepatica* burdens in treated versus untreated animals. The following equation expresses the percent efficacy (*% E*) of a drug treatment against *F. hepatica* (*F.h.*) in a single treatment group (*T*) when compared with an untreated control (*C*).

$$\%\,E = \frac{\text{Mean of } F.h. \text{ in } C \text{ - mean of } F.h. \text{ in } T}{\text{Mean of } F.h. \text{ in } C} \cdot 100$$

The geometric mean was used as it most accurately represents the distribution of parasite populations within each group (Wood et al., 1995).

Analytical procedures

Plasma sample extraction: ABZ and its metabolites were extracted from plasma and quantified by HPLC as previously described (Alvarez et al, 2008). The HPLC was a Shimadzu 10 A System (Kyoto, Japan), which include a gradient pump, a UV detector set at 292 nm, an autosampler and a controller (Shimadzu Class LC10, Kyoto, Japan). Analytes were identified by the retention times of pure reference standards. Retention times for ABZSO, $ABZSO_2$, OBZ, and ABZ were 5.32, 7.24, 9.55, and 11.14 min, respectively. There was no interference of endogenous compounds in the chromatographic determinations. Calibration curves for each analyte were prepared by least squares linear regression analysis, which showed correlation coefficients between 0.996 and 0.999. The absolute recovery of drug analytes from plasma was calculated by comparison of the peak areas from spiked plasma samples with the peak areas resulting from direct injections of standards in mobile phase. Mean absolute recoveries and coefficient of variations (CV) within the concentration range between 0.1 and 4 μg/ml (triplicate determinations) were 91.3% (CV: 6.51%) (ABZ), 89.2% (CV: 5.40%) (ABZSO) and 92.2% (CV: 6.72%) ($ABZSSO_2$). Precision (intra- and inter-assay) was determined by analysing replicates of fortified plasma samples (n= 5) with each compound at three different concentrations (0.1, 0.5 and 1 μg/ml). CV ranged from 3.50 to 12.5%. The limit of detection (LOD) was estimated by integrating the baseline threshold at the retention time of each compound in five non-spiked plasma samples. The LOD was defined as the mean 'noise'/internal standard peak area ratio plus 3 standard deviations (SD). The limit of quantification (LOQ) was defined as the lowest measured concentration with a CV <20% and accuracy of ±20%

and an absolute recovery ≥70%. The LOQ defined for the three molecules assayed was 0.1 µg/ml. Values below LOQ were not included in the pharmacokinetic analysis.

Pharmacokinetic analysis of the data

Noncompartmental pharmacokinetic calculations for the concentration versus time curves for ABZ metabolites in plasma for each individual animal after the different treatments were conducted using the PK Solution 2.0 software (Summit Research Services, CO, USA). The observed peak concentration (C_{max}) and time to peak concentration (T_{max}) were read from the plotted concentration-time curve of each analyte. The elimination (T½el) half life was calculated as ln2/β, where ß represent the terminal slope (h^{-1}). The area under the concentration time-curve (AUC) was calculated by the trapezoidal rule (Gibaldi and Perrier, 1982) and further extrapolated to infinity by dividing the last experimental concentration by the terminal slope (β). Statistical moment theory was applied to calculate the mean residence time (MRT) for metabolites in plasma, as follows: MRT= AUMC/AUC, where AUC is as defined previously and AUMC is the area under the curve of the product of time and the plasma drug concentration versus time from zero to infinity (Gibaldi and Perrier, 1982).

Statistical analysis of the data

Pharmacokinetic parameters are presented as mean ± SD. Fluke counts in each experimental group within each experiment were compared by non parametric test (Mann-Whitney Test). A value of $P<0.05$ was considered statistically significant.

Results

Table 1 shows the parasite counts and the clinical efficacy (%) for ABZ and TCBZ against the CEDIVE isolate of *F. hepatica* in sheep. Efficacy values from 29% (ABZ) and 100 % (TCBZ) were observed. No statistical differences ($P>0.05$) were observed in fluke counts between ABZ treated and untreated control groups. Furthermore, a significantly ($P<0.05$) decrease in fluke counts were observed in TCBZ treated animals, in comparison to the counting in untreated animals.

ABZSO and $ABZSO_2$ were the only analytes recovered in plasma after the i.r. administration of ABZ. The active ABZSO was the main metabolite measured in plasma up to 48 h post-treatment. The comparative mean (± SD) plasma concentration profiles of ABZSO and $ABZSO_2$ obtained after the i.r. administration of ABZ are shown in Figure 1. The plasma disposition kinetics data for ABZSO and $ABZSO_2$ after i.r. administration of ABZ are summarized in Table 2. The plasma exposure (expressed as AUC and Cmax) of ABZ metabolites observed in the ABZ treated animals (Experiment 1), was in agreement with previous data obtained in our laboratory (Table 3). The ABZSO AUC value obtained after ABZ administration in the current experiment (41.1 ± 13.9 μg.h/ml) was similar to that obtained by Alvarez et al, 1997 (41.8 ± 2.28 μg.h/ml), Alvarez et al, 1999 (46.0 ± 5.72 μg.h/ml) and Moreno et al., 2004 (31.2 ± 5.43 μg.h/ml), in which the reference formulation of ABZ (Valbazen®, ABZ 10%, Pfizer Animals Health, Argentina) was used. A similar pattern was observed for the Cmax parameter.

Discussion

The results reported here clearly demonstrate that the CEDIVE isolate of *F. hepatica* behaves as resistant to ABZ and susceptible to TCBZ. Interestingly, in the farm where the CEDIVE isolate of *F. hepatica* was obtained, anthelmintic treatments are only addressed to control GI nematodes. However, it seems clear that the high selection pressure exerted by this practice has contributed to the development of resistance not only in nematodes but even so in liver flukes.

The manufacturing process, the quality of the active ingredient/excipients and their long standing stability, among other factors involved in the production of generic formulations (suspensions), may substantially affect the drug dissolution process and its consequent GI absorption, which in turns could affect drug effectiveness. In fact, factors related to the quality of the active ingredient have been associated with therapeutic failure of generic rafoxanide formulations against *Haemonchus contortus* in sheep (Van Wyk *et al.*, 1997). Furthermore, it has been recently demonstrated that different generic ABZ formulations commercialized in Uruguay could not be assessed as bioequivalent to the pioneer preparation (Suarez et al., 2011). In the current work, the plasma drug exposure of ABZ metabolites observed after its administration as a "generic" formulation (Baxen 3.8%®), resulted similar to that reported after the administration of the reference formulation (Valbazen®) in sheep at the same dose. The AUC and Cmax values were in the range of those reported by Alvarez et al, 1997; 1999 and Moreno et al., 2004. These results discard the fact that the observed ABZ failure to control liver flues may have given by a poor quality formulation

resulting in reduced drug systemic exposure. This was not the case in the work reported here.

The intrinsic anthelmintic action of BZD compounds relies on a progressive disruption of basic cell functions as a result of their binding to parasite β-tubulin and depolimerization of microtubules (Lacey, 1988). BZD resistance in nematodes has been linked to the loss of high-affinity binding to tubulin (Lubega and Prichard, 1991a) and an alteration of the β-tubulin isoform pattern (Lubega and Prichard, 1991b), correlated with a conserved mutation at amino acid 200 (phenylalanine to tyrosine: F200Y) in tubulin isotype 1 (Kwa et al., 1994). While experimental data supports a microtubule-based action for TCBZ (reviewed by 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 fact, the accumulated *in vitro* data demonstrate that at least two mechanisms appear to be implicated in TCBZ resistance in *F. hepatica*: increased drug efflux and enhanced oxidative metabolism (Robinson et al., 2004; Alvarez et al., 2005a; Mottier et al., 2006; Devine et al., 2009; Devine et al., 2010).
A previous study has shown that ABZ is active against a TCBZ-resistant isolate of *F. hepatica* (Coles and Stafford, 2001). The greater trans-tegumental diffusion capability of ABZ compared to TCBZ under *ex vivo* conditions, may account for its efficacy pattern against TCBZ-resistant flukes (Alvarez et al., 2005b). However, the CEDIVE isolate results resistant to ABZ and susceptible to TCBZ. These results may indicate that a different mechanism could be implicated in the development of resistance to each compound, it is likely that TCBZ may target a molecule other than ß-tubulin, which

would explain why ABZ continues to act against TCBZ-resistant flukes. To complicate the picture, resistance to ABZ and TCBZ in *F. hepatica* has been reported in Spain (Alvarez-Sanchez et al., 2006). It is, therefore clear, that the mechanism involved on ABZ resistance in *F. hepatica* need to be elucidated.

Conclusions

The data reported here indicate that the CEDIVE isolate of *F. hepatica* is resistant to ABZ and susceptible to TCBZ. The fact that the CEDIVE isolate is maintained under laboratory conditions, may help to further understand the mechanism(s) of ABZ resistance in *F. hepatica*.

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Figure captions

Figure 1: Comparative mean (±SD) plasma concentration profiles (n= 4) for albendazole sulphoxide (ABZSO), after the administration of albendazole (Baxen3.8%®, 7.5 mg/kg) to sheep infected with *Fasciola hepatica*.

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Figures

Figure 1. ABZSO and ABZSO2 plasma concentrations in*Fasciola hepatica*-infected sheep (mean and standard deviations).

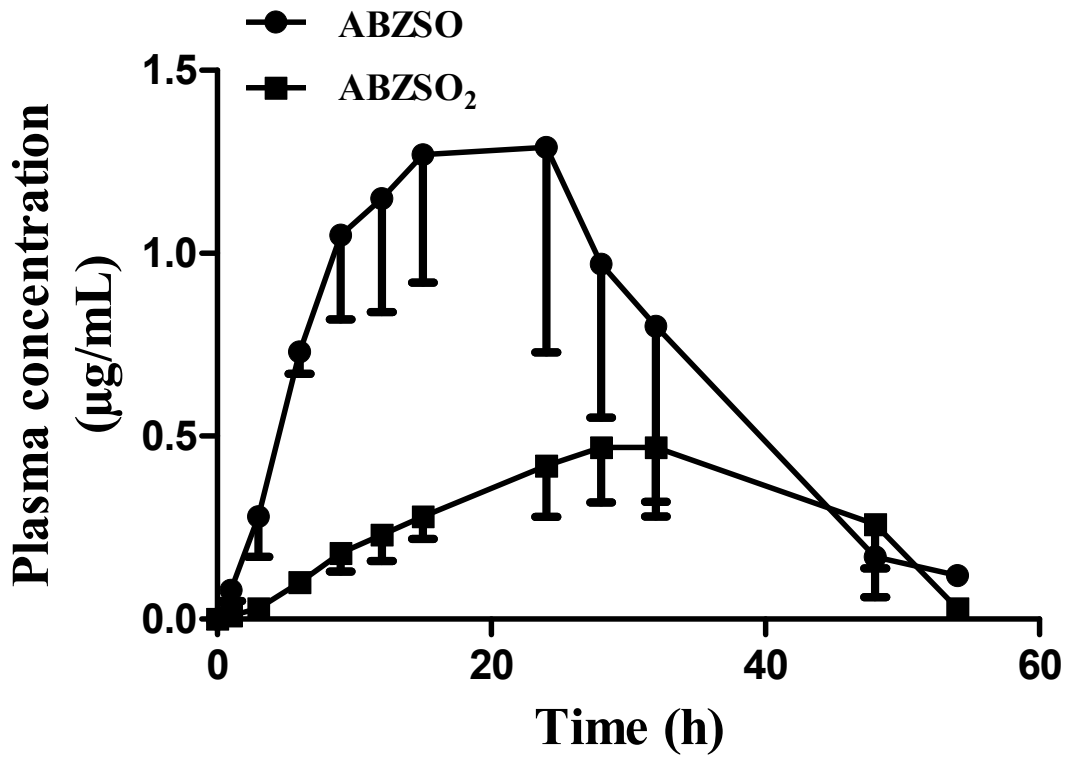

Table 1. Individual and mean fluke counts and clinical efficacy (%) against the CEDIVE *Fasciola hepatica* isolate, obtained after the administration of albendazole (ABZ, 7.5 mg/kg, i.r.) or triclabendazole (TCBZ, 10 mg/kg, i.r.) in sheep.

Animal	Control Group	ABZ Group	Control Group	TCBZ Group

# 1	112	60	27	0
# 2	67	25	23	0
# 3	43	69	15	0
# 4	67	55	29	0
Arithmetic mean	72.3	52.3	23.5	0
Efficacy*	-	**29 %**		**100%**

* The efficacy was calculated using geometric means.

Table 2. Plasma pharmacokinetic parameters (mean ± SD) for albendazole sulphoxide (ABZSO) and albendazole sulphone ($ABZSO_2$) obtained after the intraruminal (i.r.) administration of albendazole (ABZ, 7.5 mg/kg) to *Fasciola hepatica* infected sheep.

PHARMACOKINETIC PARAMETERS	**ABZSO**	**$ABZSO_2$**
Cmax (µg/mL)	1.40 ± 0.47	0.50 ± 0.10
Tmax (h)	16.5 ± 5.20	30.0 ± 2.30
AUC_{0-t} (µg.h/mL)	41.1 ± 13.9	15.9 ± 5.30
T½el (h)	8.70 ± 0.80	7.30 ± 0.60
MRT (h)	23.1 ± 2.14	29.6 ± 1.80

Table 3. Comparative peak plasma concentration (Cmax)and area under the concentration vs time curve (AUC) for albendazole sulphoxide (ABZSO), obtained after the i.r. administration of albendazole (7.5 mg/kg) as a 3.8% formulation (Baxen 3.8%®, Tecnofarm, Argentina, current work) or as a 10% formulation (Valbazen, Pfizer Animal Health, Argentina, reference formulation)(works from Alvarez et al., 1997; 1999 and Moreno et al., 2004) to sheep.

ABZSO PK Parameter	**Current work**	***Alvarez et al., 1997***	***Alvarez et al., 1999***	***Moreno et al., 2004***
Cmax (µg/ml)	1.40 ± 0.47	1.76 ± 0.16	1.92 ± 0.26	1.23 ± 0.24
AUC (µg.h/ml)	41.1 ± 13.9	41.8 ± 2.28	46.0 ± 5.72	31.2 ± 5.43