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Assessment of the pharmacological interactions between the nematodicidal fenbendazole and the flukicidal triclabendazole: In vitro studies with bovine liver microsomes and slices

P. Viviani[1] | A. L. Lifschitz[1] | M. L. Maté[1] | J. P. García[2] | C. E. Lanusse[1] | G. L. Virkel[1]

[1]Facultad de Ciencias Veterinarias, Laboratorio de Farmacología, Centro de Investigación Veterinarias de Tandil (CIVETAN-CONICET-CICPBA), Universidad Nacional del Centro de la Provincia de Buenos Aires (FCV-UNCPBA), Tandil, Argentina

[2]Facultad de Ciencias Veterinarias, Área Clínica de Grandes Animales, Universidad Nacional del Centro de la Provincia de Buenos Aires (FCV-UNCPBA), Tandil, Argentina

Correspondence
Guillermo L. Virkel, Facultad de Ciencias Veterinarias, Laboratorio de Farmacología, Centro de Investigación Veterinarias de Tandil (CIVETAN-CONICET-CICPBA), Universidad Nacional del Centro de la Provincia de Buenos Aires (FCV-UNCPBA), Tandil, Argentina.
Email: gvirkel@vet.unicen.edu.ar

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Parasitic diseases have a significant impact on livestock production. Nematodicidal drugs, such as fenbendazole (FBZ) or its oxidized metabolite oxfendazole (OFZ), can be used along with the trematodicidal triclabendazole (TCBZ), to broaden the spectrum of anthelmintic activity. However, co-exposure to these compounds could lead to drug–drug (D-D) interactions and eventually alter the clinical profile of each active principle. The aim of this study was to assess the presence of such interactions by means of two in vitro models, namely bovine liver microsomal fractions and bovine precision-cut liver slices (PCLSs). To this end, an in vitro assessment involving incubation of FBZ and TCBZ or a combination of FBZ and TCBZ was carried out. Results with microsomal fractions showed a 78.4% reduction (p = .002) in the rate of OFZ production upon co-incubation, whereas the sulfoxide metabolite of TCBZ (TCBZSO) exhibited a decreasing tendency. With PCLS, OFZ accumulation in the incubation medium increased 1.8-fold upon co-incubation, whereas TCBZSO accumulation decreased by 28%. The accumulation of FBZ and OFZ in the liver tissue increased upon 2-hr co-incubation, from 2.1 ± 1.5 to 18.2 ± 6.1 (p = .0009) and from 0.4 ± 0.1 to 1.3 ± 0.3 nmol (p = .0005), respectively. These results confirm the presence of D-D interactions between FBZ and TCBZ. Further studies are needed to determine the extent of involvement of drug-metabolizing enzymes and membrane transporters in interactions between compounds largely used in livestock production systems.

1 | INTRODUCTION

Parasite infections in cattle pose a significant threat to animal health and productivity in grazing systems worldwide. Many control programs in commercial farms rely heavily on the use of anthelmintics, that is benzimidazoles, imidothiazoles/tetrahydropyrimidines, and macrocyclic lactones (Sutherland & Leathwick, 2011; Waller, 2006). However, resistance to one or more of these chemical groups has emerged as a result of high selection pressure on parasite populations. Current guidelines suggest a variety of strategies as an attempt to overcome this situation, including identifying and avoiding high-risk management practices, maintaining *refugia* of susceptible parasites within the existing populations (Leathwick & Besier, 2014), and choosing optimal drugs or drug combinations (Geary et al., 2012). The latter has been suggested as valuable tools to either delay or control resistance, or to expand the spectrum of efficacy of individual molecules.

Although combinations typically exploit the effects of two active moieties with differing mechanisms of action, concurrent exposure to same-class molecules can also display advantageous properties, both at a pharmacodynamic and a pharmacokinetic level (Lanusse, Alvarez, & Lifschitz, 2014). Treatments combining two benzimidazole anthelmintics (BZDs) have exhibited such effects in a variety of situations. In livestock, BZDs including fenbendazole (FBZ), triclabendazole (TCBZ) and albendazole (ABZ) are routinely used to treat and prevent a variety

of endoparasitosis (Campbell, 1990). FBZ, its active metabolite oxfendazole (OFZ), and ABZ are indicated for the treatment of gastrointestinal and respiratory worms, whereas TCBZ is used against both juvenile (immature) and adult specimens of the liver fluke *Fasciola hepatica* (Barrera et al., 2012). Therefore, combinations of TCBZ and FBZ, OFZ or ABZ may result in an increased spectrum of efficacy compared with single-drug dosages. In this regard, the combination of TCBZ with either FBZ or OFZ has exhibited enhanced efficacy against *F. hepatica*, in particular against immature stages (Ali, Bogan, Marriner, & Richards, 1986; Foreyt, 1988).

Both FBZ and TCBZ undergo extensive hepatic metabolism, ultimately leading to the formation of S-oxidized metabolites, namely OFZ and TCBZ sulfoxide (TCBZSO), respectively. In turn, their S-oxides undergo a second step of S-oxidation yielding virtually inactive sulfone metabolites, FBZ sulfone ($FBZSO_2$), and TCBZ sulfone ($TCBZSO_2$), respectively. Both FBZ and TCBZ, as well as their sulfoxide metabolites, are cytochrome P450 (CYP) and flavin-monooxygenase (FMO) substrates (Virkel, Lifschitz, Sallovitz, Pis, & Lanusse, 2004, 2006), and consequently, a metabolic interaction upon their co-administration is likely to occur. Altered pharmacokinetic profiles due to such interactions can eventually lead to variation in drug concentrations in the biophase around parasites and, ultimately, alter the clinical efficacy of the co-administered moieties.

From a quantitative viewpoint, metabolism of BZDs takes place mostly in the liver (Lanusse & Prichard, 1993). Although other tissues also display metabolic ability, in vitro models have generally focused on hepatic metabolism to elucidate pathways of drug biotransformation. Traditional models include primary hepatocytes, immortalized cell lines, and microsomal fractions (Vermeir et al., 2005). More recently, precision-cut liver slices (PCLSs) have gained popularity as a reliable, accurate model preserving tissue spacial and functional relationships (Elferink et al., 2011). PCLSs are similar to other explants in that tissue architecture and cell integrity are maintained, as well as the bipolarity of hepatocytes and the ratio of parenchymal to nonparenchymal cells (Gebhardt et al., 2003). As a result, cell populations and their relationships with each other and the extracellular scaffolding system are preserved. This is especially useful for the study of metabolic pathways of both endogenous and exogenous compounds, including metabolic drug–drug (D-D) interactions (Boess, 2003; Elferink et al., 2008).

Treatment combining either FBZ or OFZ together with TCBZ could be a strategy for widening the spectrum of efficacy. With this purpose, an oral drench containing a combination of TCBZ and OFZ is marketed in several countries, and high efficacy against early immature liver flukes has been achieved in sheep receiving this formulation (Martin, Chambers, & Hennessy, 2009). However, to the best of our knowledge, there is no information on the potential D-D interactions between these molecules and the consequences on parasite exposure and drug final concentrations both within the body and as environmental residues. Concerns about the potential impact of parmacokinetic/pharmacodynamic interactions on environment, animal, and food safety warrant further research, as suggested by the WAAVP Guidelines (Geary et al., 2012). This study aims at helping fill this void by investigating the potential metabolic interactions occurring in vitro between FBZ and TCBZ in microsomal fractions and PCLS from cattle.

2 | MATERIALS AND METHODS

2.1 | Chemicals

Salts (KCl, NaCl, $Cl_2Ca{\bullet}2H_2O$, $Cl_2Mg{\bullet}6H_2O$, $NaHCO_3$, Na_2CO_3, Na_2HPO_4, $NaH_2PO_4{\bullet}H_2O$, K_2HPO_4, KH_2PO_4, CH_3COONH_4, and NaOH) and HPLC grade acetonitrile (ACN) were purchased from Baker Inc. (Phillipsburg, NJ, USA). Williams' Medium E (WME), ampicillin, EDTA, N-ethylmaleimide, $HClO_4$, butylated hydroxytoluene, trichloroacetic acid (TCA), and nicotinamide adenine dinucleotide phosphate ($NADP^+$) were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Bovine insulin was purchased from Beta Laboratories (Buenos Aires, Argentina). Gentamicin was obtained from Parafarm (Buenos Aires, Argentina). Ascorbic acid was from Anedra (Buenos Aires, Argentina). D-Glucose and HCO_3Na were purchased from Biopack (Buenos Aires, Argentina). Fungizone (amphotericin B) and O-pthalaldehyde were from Thermo Fisher Scientific (Carlsbad, CA, USA). Glucose-6-phosphate and glucose-6-phosphate dehydrogenase were purchased from Roche Applied Science (Buenos Aires, Argentina). Reference standards of fenbendazole (FBZ), oxfendazole (OFZ), and fenbendazole sulfone ($FBZSO_2$) were purchased from Toronto Research Chemicals (ON, Canada), whereas triclabendazole (TCBZ), triclabendazole sulfoxide (TCBZSO), and triclabendazole sulfone ($TCBZSO_2$) were obtained from Novartis Argentina (Buenos Aires, Argentina). Oxibendazole (OBZ) was from GlaxoSmithKline (PA, USA).

2.2 | Animals

For preparation of both microsomal fractions and PCLS, bovine liver samples were obtained from a local slaughterhouse (Mirasur SA, Tandil, Argentina) located 16 km away from the laboratory facilities. Procedures and management protocols for all animal species were carried out in accordance with the Animal Welfare Policy (Academic Council Resolution 087/02) of the Faculty of Veterinary Medicine, Universidad Nacional del Centro de la Provincia de Buenos Aires (UNCPBA), Tandil, Argentina (Internal Protocols: 02/2011 and 12/2013; approval dates: March 1st, 2012 and October 30th, 2013, respectively). In the slaughterhouse, animals were sacrificed in agreement with this institutional animal welfare policy.

Liver samples of the caudate lobe were obtained from fifteen Aberdeen Angus/Hereford crossbreed steers with an approximate weight of 350 kg. For preparation of microsomal fractions, liver samples from six (n = 6) animals, weighting 8–10 g, were used. Tissue samples were gently rinsed with cold KCl 1.15%, wrapped in aluminum foils, and refrigerated at 4°C during their transport to the laboratory facilities.

For PCLS preparation, liver samples from nine (n = 9) animals were obtained. Immediately after sacrifice, a sample (roughly 20 g) from the

caudate lobe was excised, rinsed with ice-cold 1.15% KCl, and immersed in Krebs buffer (1 mM $NaH_2PO_4 \bullet H_2O$; 2.5 mM $Cl_2Ca \bullet 2H_2O$; 4.7 mM KCl; 1.1 mM $Cl_2Mg \bullet 6H_2O$; 0.004 mM EDTA; 11 mM glucose; 119 mM NaCl, 25 mM Na_2CO_3; 0.11 mM ascorbic acid) at 4°C. Containers were capped, cooled in ice, and transported to the laboratory within 30–40 min for subsequent procedures.

2.3 | Preparation and incubation of microsomal fractions

Microsomal fractions were prepared as described by Maté, Virkel, Lifschitz, Ballent, and Lanusse (2008). TCBZ and FBZ were incubated either alone or in combination (TCBZ + FBZ) with a reaction mixture containing 1 mg/ml microsomal protein in a final volume of 500 µl phosphate buffer 0.1 M (pH 7.4). Each substrate (FBZ, TCBZ, and TCBZ + FBZ), dissolved in 10 µl of methanol, was added to this mixture, which was then allowed to equilibrate for 1 min at 37°C. All of the control and combined incubations were carried out in duplicate, and the final concentration of the substrates in the assay mixtures was 50 µM. Each metabolic reaction was started by the addition of a NADPH-generating system ($NADP^+$ 0.32 mM, glucose-6-phosphate 6.4 mM, $MgCl_2$ 5 mM, EDTA 0.8 mM, and 1.25 U glucose-6-phosphate dehydrogenase in 0.1 M phosphate buffer, pH 7.4). Blank samples containing all of the aforementioned components except the NADPH-generating system were also incubated to determine nonenzymatic metabolism. Incubations were carried out in an oscillating water bath under aerobic conditions and lasted for 15 min, after which the reaction was stopped by the addition of 300 µl ACN. The samples were then stored at −4°C until analysis.

2.4 | Preparation and culture of PCLS

Precision-cut liver slices were obtained as previously described by Maté et al. (2015). Following preparation, slices were immediately transferred to a Petri dish containing ice-cold oxygenated WME and then to 12-well culture plates (Corning Incorporated, Corning, NY, USA). Each well contained one slice and 1.3 ml of WME. The plates were incubated in a container placed on an orbital shaker (Ferca, Buenos Aires, Argentina) set at 60 rpm and maintained at 37°C under a humidified atmosphere of 95% O_2:5% CO_2. After a 1 hr preincubation (t = 0), WME was completely replaced by fresh medium fortified with 50 µM of FBZ, TCBZ or a combination of TCBZ and FBZ. All of the aforementioned compounds were previously dissolved in methanol and then added to aliquots of the WME. The final solvent concentration in the culture medium was 2%.

To determine accumulation rates in WME, the culture medium in each well was sampled by taking 150 µl aliquots at 1, 2, 4, 6, 8, and 12 hr, followed by reposition with drug-free WME. Additional wells were incubated with each drug or combination but without slice and sampled at the same time points to evaluate the persistence of the drugs in the medium throughout the experiments. No tissue samples were collected during this experiment. Control and combined incubations were performed in triplicate. Additionally, a different set of wells was incubated with each drug or combination to a final time point of either 2 or 6 hr, after which both the slice and the WME were collected and stored at −20°C until analysis. In the latter experiment, control and combined incubations were carried out in duplicate.

2.5 | Viability assessment of PCLS

For each assay, viability was assessed by means of three indicators of tissue integrity, that is histopathological evaluation and two biochemical determinations, reduced glutathione levels and intracellular potassium (K_i^+) content. Briefly, slices for histopathological evaluation were sampled at 0- and 12-hr incubation. Additionally, freshly prepared slices were also collected. The samples were stored at room temperature in 10% buffered formalin. After 24 hr, they were dehydrated with ethanol and embedded in paraffin blocks, which were then cut to 5-µm thick cross-sections by means of a rotary microtome. Following rehydration in distilled water and staining with haematoxilin and eosin (HE), evaluation was carried out by a trained veterinary pathologist, who was blind to the identity of each sample. Hepatocyte loss, hydropic degeneration, and hepatocyte fragmentation were assessed as morphological indicators. For each individual indicator, the degree of severity was scored using a subjective scale comprising degrees 0 (absence of histological damage), 1 (mild), 2 (moderate), and 3 (severe damage).

For both biochemical determinations, five individual slices were pooled. Four pools were prepared for the initial (t = 0), intermediate (t = 6), and final (t = 12) times of incubation. Slices for K_i^+ content were additionally washed with NaCl 0.9% after harvesting and stored at 4°C until analysis. Pools for determination of glutathione and K_i^+ content were processed as previously described (Viviani et al., 2017). Briefly, thawed slices were homogenized in Tris-HCl buffer (50 mM, pH 7.4) with KCl 0.15 M and butylated hydroxytoluene18 µM and subsequently centrifuged. The resulting supernatant was assayed for reduced (GSH) following a modification of the procedure described by Hissin and Hilf (1976). GSH contents were measured by means of a RF-5301 PC spectrofluorophotometer (Shimadzu Corporation, Kyoto, Japan). Results are expressed as pmol of GSH per mg of slice. Slices for determination of K_i^+ content were homogenized in 1 ml distilled water, and protein was precipitated by the addition of 20 µl $HClO_4$. After centrifugation, K_i^+ was measured in the supernatant following the methodology described by Fisher, Hasal, Lipscomb, Gandolfi, and Brendel (2002). Samples were assayed with an atomic absorption spectrophotometer (GBC Scientific Equipment, Braeside, Australia). Results are expressed as nmol of K_i^+ per mg of slice.

2.6 | Chromatographic analysis

Sample analysis by high-performance liquid chromatography (HPLC) was carried out as described in Viviani et al. (2017). Briefly, an internal standard (OBZ 0.25 mM in methanol) was added to inactivated incubation mixtures (liver microsomes), WME samples (20 µl

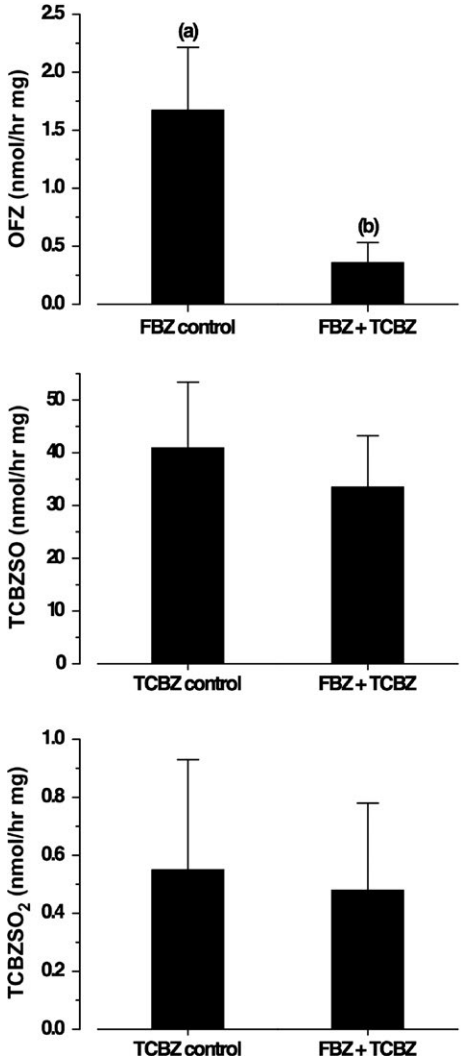

FIGURE 1 Biotransformation of fenbendazole (FBZ) and triclabendazole (TCBZ) by cattle liver microsomes. Both anthelmintics were incubated alone (control incubations) and in combination. The upper panel shows the effect of TCBZ on the rate of production of oxfendazole (OFZ) from FBZ. Middle and lower panels show the effect of FBZ on the rates of production of triclabendazole sulfoxide (TCBZSO) and sulfone ($TCBZSO_2$), respectively. Data are the mean ± *SD* of six liver microsomal preparations. Values with different superscripts are significantly different ($p < .05$)

of internal standard solution), and PCLS (10 µl of internal standard solution). Incubated microsomal samples were shaken for 10 s and centrifuged at 9,000 *g* for 15 min at 10°C. Supernatants were directly injected into the HPLC system. WME samples were mixed with 100 µl of ACN, shaken, and centrifuged at 9,000 *g* for 15 min at 10°C. Tissue samples (PCLSs) were mixed with 1 ml ACN and then subjected to manual homogenization. Homogenized samples were shaken for 15 min, centrifuged at 12,000 *g* for 15 min at 10°C, and then evaporated to dryness. The supernatants obtained from the incubation mixtures (microsomes) and from WME samples were injected into the HPLC system without further processing, whereas evaporated tissue samples were resuspended in 300 µl mobile phase prior to injection.

Samples were analyzed for TCBZ, TCBZSO, $TCBZSO_2$, FBZ, OFZ, $FBZSO_2$, and OBZ. A volume of 50 µl was injected through an auto sampler (Shimadzu SIL-10 A Automatic Sample Injector) into a Shimadzu 10 A HPLC system (Shimadzu Corporation, Kyoto, Japan) fitted with a Kromasil C18 reverse-phase column (250 × 4.6 mm, particle size 5 µm, Eka Chemicals, USA) and a UV detector (SPD-10 A UV detector, Shimadzu) set at a wavelength of 292 nm. The mobile phase consisted of 42% ACN and 58% ammonium acetate (0.025 M, pH 5.3). The chromatographic conditions were as previously reported (Virkel et al., 2004), with slight modifications made to the elution gradient. The analytes were identified by matching their retention times with those of pure reference standards. Chromatographic peak areas were determined using the integrator software (LCsolution; Shimadzu Corporation, Kyoto, Japan) of the HPLC system. The procedures for quantification of TCBZ, FBZ and their metabolites were validated as described by Virkel et al. (2004, 2006).

2.7 | Statistical analysis

Data are expressed as mean ± standard deviation (*SD*). Results of assays with microsomal fractions are expressed as nmol per mg of microsomal protein per hour (nmol/hr.mg protein). Results of incubations with PCLS are expressed as nmol per mg of tissue (nmol/mg tissue), whereas accumulation rates in WME are expressed as nmol/hr. Linearity was determined by least-squares regression analysis. Results of biochemical determinations are expressed as nmol/mg of tissue for K_i^+ and as pmol/mg of tissue for GSH. Biochemical measurements of GSH and K_i^+ across incubation times were compared by means of one-way ANOVA or nonparametric Kruskal–Wallis test depending on whether the standard deviations were similar or not between time groups.

Statistical analysis of the FBZ and TCBZ interaction was performed using R Statistical Software, version 3.3.2 (R Core Team, 2016) and Instat 3.0 (Graph Pad Software Inc., San Diego, CA, USA). The Shapiro–Wilks method was used as a normality test. Comparisons were performed with a Student's *t* test with Welch correction. Differences were considered statistically significant for *p* values below .05.

3 | RESULTS

3.1 | Assays in microsomal fractions

Both FBZ and TCBZ were metabolized in bovine liver microsomal fractions. Figure 1 shows the rates of production of the oxidized metabolites of FBZ and TCBZ when both anthelmintics were incubated alone or in combination. The co-incubation of FBZ with TCBZ resulted in a net reduction of 78.4% ($p = .002$) in the rate of production of OFZ (Figure 1, upper panel). In contrast, co-incubation did not alter the rates of production of TCBZ metabolites, namely TCBZSO and $TCBZSO_2$, compared with the controls.

3.2 | Viability assessment of PCLS

The viability of PCLS was assessed by histomorphological evaluation (Figure 2 and Table 1) and K_i^+ and GSH contents (Figure 3). Structural

differences were observed between fresh slices and those incubated for 12 hr. Hepatocyte loss and fragmentation were the most prominent findings. PCLS also exhibited a mild degree of hydropic degeneration after 12 hr of incubation, but no necrosis was present. In terms of functional integrity, no overall differences were observed. GSH and K_i^+ levels (Figure 3) decreased slightly over time, although this trend was not statistically significant.

3.3 | Assays with PCLS

Precision-cut liver slices were able to metabolize FBZ and TCBZ mainly to their respective S-oxidized metabolites, as shown in Figure 4. Results are shown up to 6 hr for OFZ and 8 hr for TCBZSO, as this was the time frame during which a linear accumulation of both metabolites was observed. When FBZ was incubated alone, OFZ accumulated in the culture medium at a mean rate of 0.41 ± 0.17 nmol/hr. The rate of OFZ accumulation rose from 1.8-fold (p = .008) to 0.73 ± 0.27 nmol/hr in the presence of TCBZ. Trace amounts of $FBZSO_2$ were measured in incubations with FBZ (data not shown).

The pattern of TCBZSO accumulation changed in the presence of FBZ. Compared with control incubations (0.32 ± 0.08 nmol/hr), co-incubation resulted in a 28% decrease (p = .004) in the accumulation rate of TCBZSO to 0.23 ± 0.07.

Absolute accumulation of substrates and metabolites was quantified in bovine PCLS at both 2- and 6-hr incubation, as shown in Tables 2 and 3. Substrate accumulation was roughly similar under all tested conditions. The only exception was FBZ (Table 2), which yielded a significantly lower (p = .0009) total accumulation at 2 hr in control incubations (2.2 ± 1.5 nmol) compared with co-incubation with TCBZ (18.2 ± 6.1 nmol). In incubations with FBZ (Table 2), accumulation of OFZ exhibited a similar tendency to that observed in WME, with a 3.53-fold increase (p = .0005) after 2-hr co-incubation (1.3 ± 0.3 nmol) compared with controls (0.4 ± 0.1 nmol). Although a similar tendency was observed at 6-hr incubation, with more metabolite formed under co-incubation (1.0 ± 0.7 nmol) compared with controls (0.7 ± 0.1 nmol), differences were not statistically significant.

In contrast to the situation observed in the WME, no significant changes were observed in tissue accumulation of TCBZSO under co-incubation of TCBZ with FBZ (Table 3).

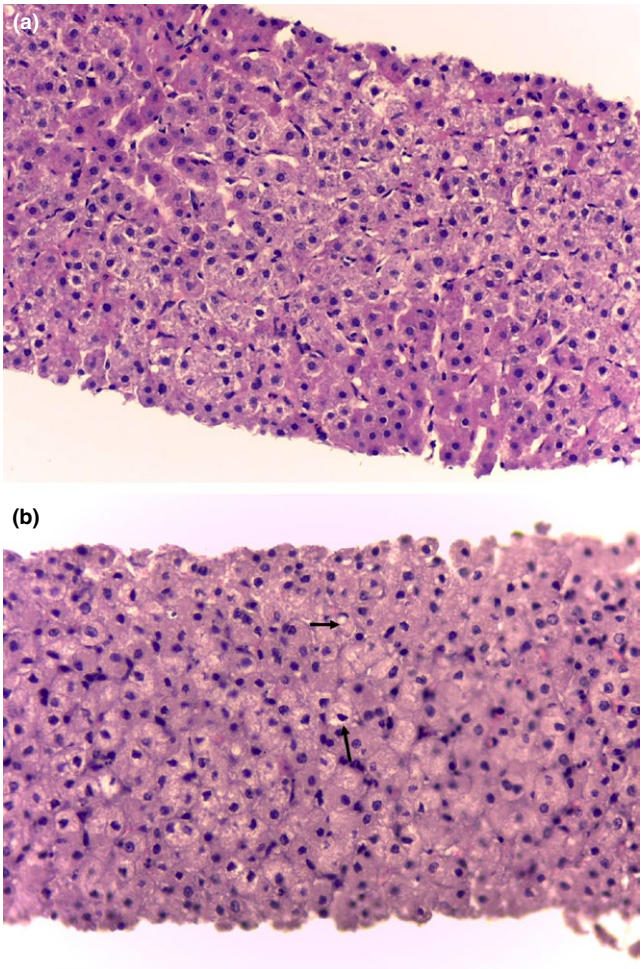

FIGURE 2 Histopathology of bovine precision-cut liver slices (PCLSs). Tissue sections were stained with hematoxylin and eosin, and photomicrographs were taken with 400× magnification. (a) fresh PCLS; (b) PCLS, 12-hr incubation showing mild hydropic degeneration (arrows)

4 | DISCUSSION

The clinical profile of a therapeutic agent can be affected by several factors, leading either to reduced or enhanced activity, or even to toxicity (Brandon, Raap, Meijerman, Beijnen, & Schellens, 2003). Concomitant use of two or more molecules can lead to interactions at a pharmacokinetic or at a pharmacodynamic level, or both. The pharmacokinetic mechanisms of D-D interactions usually result in altered concentrations of one or both drugs within the body, thus, affecting the expected therapeutic outcome (Mozayani & Raymon, 2004). In the treatment of parasitic disease, underexposure of helminths to the administered molecules can result in therapeutic failure. The selection pressure that suboptimal concentrations exert on helminth populations, which in turn favors the survival and multiplication of resistant strains, poses an additional threat (Furtado, de Paiva Bello, & Rabelo, 2016).

Chemotherapy remains the cornerstone of parasite control programs (Waller, 2006). The risk of therapeutic failure warrants further research into the presence and mechanisms of interactions. The current study addresses this issue by means of two different in vitro methodologies, namely bovine microsomal fractions and PCLS. Microsomal fractions are a well-established model in metabolism research, in particular for identifying metabolites and comparing interspecies biotransformation of xenobiotic compounds (Nebbia, Dacasto, Rossetto Giaccherino, Giuliano Albo, & Carletti, 2003). Liver microsomes possess high concentrations of a variety of drug-metabolizing enzymes, including CYP and FMO (Asha & Vidyavathi, 2010), and can be prepared from a variety of tissues and species and stored for ulterior use. This method does not allow for estimations

TABLE 1 Histological evaluation of bovine precision-cut liver slices (PCLS). Each value represents the mean of six observations

Incubation time (hr)	Hydropic degeneration	Hepatocyte fragmentation	Hepatocyte loss
0 (fresh)	0	0.83	0.33
12	0.67	2.67	2

Severity was scored between 0 and 3, with 0 indicating no microscopic lesions and values between 1 and 3 indicating increasingly severe microscopic lesions.

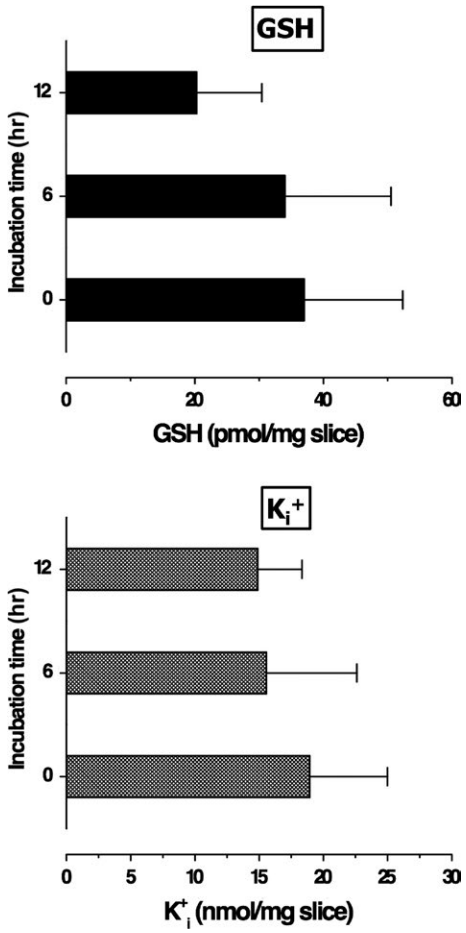

FIGURE 3 Intracellular reduced glutathione (GSH) (upper panel) and K_i^+ (lower panel) in 0-, 6-, and 12-hr cultured precision-cut liver slices. Values are expressed as pmol of GSH and nmol of K_i^+ per mg of slice. Data are the mean ± *SD* of four pools, each containing five individual slices

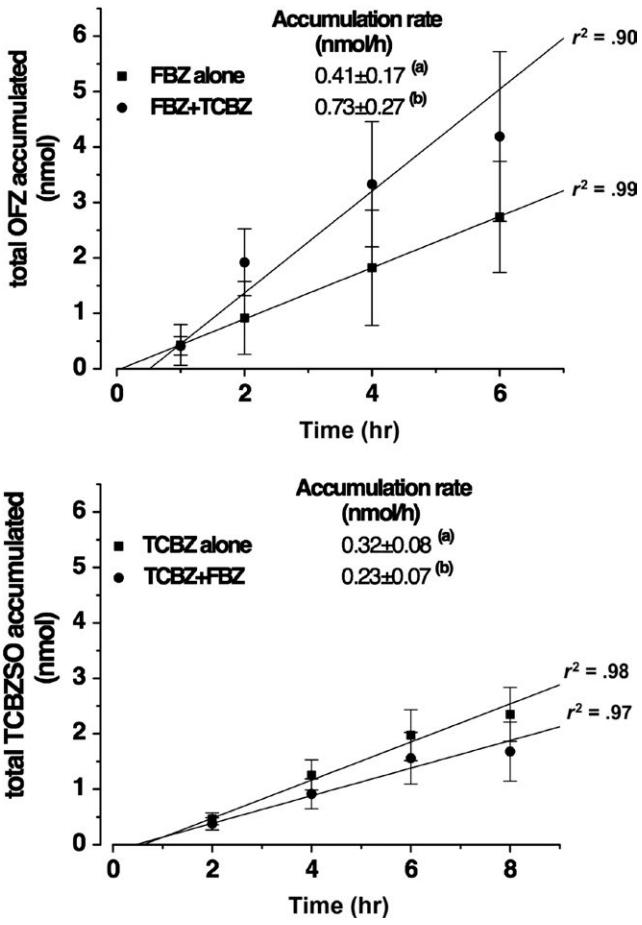

FIGURE 4 Accumulation of oxfendazole (OFZ) (upper panel) and triclabendazole sulfoxide (TCBZSO) (lower panel) in the culture medium after the incubation of cattle liver slices in the presence of fenbendazole (FBZ), triclabendazole (TCBZ) and the combination of both anthelmintics. Inserts show the accumulation rates (nmol/hr) of each metabolite. Data are the mean ± *SD* of six slice cultures. r^2: coefficients of determination for each linear regression. Values with different superscripts are significantly different ($p < .05$)

of in vivo metabolism, as some enzymatic complexes, including CYPs and UDP-glucuronosyltransferases, are over-represented, and the lack of cytosolic enzymes and cofactors prevents the characterization of alternative pathways (Asha & Vidyavathi, 2010). Incubation times longer than 2 hr are not recommended without suitable controls to check for enzyme activity. In spite of these drawbacks, microsomal fractions remain a useful model in terms of convenience and versatility, and their use is often warranted in high-throughput assays (Jia & Liu, 2007). On the other hand, PCLSs maintain functional hepatocytes with their full complement of both phase I and phase II metabolizing enzymes, as well as their transporter proteins. Viability, which is critical for the validity of incubation results, has been shown to be maintained for 12 hr under the current conditions (Viviani et al., 2017). Compared with microsomal fractions, PCLSs are particularly useful as predictors of in vivo metabolism, as reactions involving many enzymatic systems in a wide variety of intracellular locations can be assessed (Jia & Liu, 2007). In a previous work (Viviani et al., 2017), a well-characterized D-D interaction in cattle was assayed in bovine PCLS to establish the usefulness of this technique in the study of the interplay between different molecules. The present study aims at further establishing PCLS as a valid

TABLE 2 Effect of triclabendazole (TCBZ) on the accumulation of fenbendazole (FBZ) and its metabolic product oxfendazole (OFZ) in bovine precision-cut liver slices (PCLSs)

		Incubation assay	
Drug/metabolite	Time (hr)	FBZ control	FBZ + TCBZ
FBZ	2	2.1 ± 1.5	18.2 ± 6.1[a]
	6	18.6 ± 4.3	18.7 ± 11.9
OFZ	2	0.4 ± 0.1	1.3 ± 0.3[b]
	6	0.7 ± 0.1	1.0 ± 0.7

Data are the mean (±*SD*) of the total amounts (nmol) of both FBZ and OFZ measured within six PCLS.
[a]Significantly different (p = .0009) vs. FBZ control.
[b]Significantly different (p = .0005) vs. FBZ control.

TABLE 3 Effect of fenbendazole (FBZ) and oxfendazole (OFZ) on the accumulation of triclabendazole (TCBZ) and its S-oxide metabolite (TCBZSO) in bovine precision-cut liver slices (PCLS)

		Incubation assay	
Drug/metabolite	Time (hr)	TCBZ control	TCBZ + FBZ
TCBZ	2	24.7 ± 9.5	25.0 ± 5.6
	6	20.7 ± 7.1	24.5 ± 10
TCBZSO	2	12.2 ± 2.9	13.1 ± 4.0
	6	19.9 ± 4.7	18.9 ± 3.1

Data are the mean (±*SD*) of the total amounts (nmol) of both TCBZ and TCBZSO measured within six PCLS.

methodological tool to assess potential interactions between two widely used anthelmintic compounds.

Although direct comparisons between in vitro methods are difficult to assess, rough tendencies can be identified in the behavior of different moieties. The inherent features of each model can help to explain the differences observed in the current study. In microsomal fractions, co-incubation of FBZ and TCBZ resulted in a marked reduction in OFZ production. In a study using rat intestinal microsomes, competitive inhibition was identified as the mechanism explaining the inhibitory effect of FBZ on the sulfoxidation of its structural analog ABZ (Villaverde et al., 1995). Concurrent exposure of CYP and FMO to FBZ and TCBZ would thus lead both substrates to compete for their binding sites, resulting in inhibition of FBZ sulfoxidation.

In contrast to microsomal fractions, OFZ production was not diminished but increased upon co-incubation of FBZ and TCBZ in bovine PCLS. An enhanced OFZ accumulation was observed both in the culture medium and within the tissue (see Figure 4 and Table 2). In this regard, both TCBZ and TCBZSO were shown to be inhibitors of the transporters P-glycoprotein (P-gp) (Dupuy, Alvinerie, Ménez, & Lespine, 2010) and breast cancer resistance protein (BCRP) (Barrera et al., 2012), at least in murine and human cellular models. FBZ has been shown not to interact with BCRP, P-gp, or the multidrug-resistant protein 2 (MRP2) in MDCK cells transduced with human cDNAs of these transporters (Merino et al., 2005). The same study also demonstrated transport of OFZ by murine but not by human BCRP. In this regard, assays carried out by Wassermann, Halwachs, Lindner, Honscha, and Honscha (2013) showed that BCRPs from dairy cows, sheep, and goats exhibit quantifiable differences in terms of affinity to a variety of substrates, including albendazole and some antibiotics. These differences across species warrant further research on the specific involvement of each transporter in cattle. Whether or not FBZ is a substrate of these transport proteins in this species is still unknown. However, under this circumstance, an increased exposure of the parent compound to xenobiotic-metabolizing enzymes may occur, thus, leading to enhanced metabolism toward OFZ. Involvement of membrane transporters could also explain the higher intracellular accumulation of FBZ at 2 hr of incubation of FBZ in the presence of TCBZ with respect to FBZ alone (see Table 2). As membrane transport proteins are saturable (Kunta & Sinko, 2004), steady-state conditions might have been reached after only short incubation times, which could explain why differences were detected at 2-hr incubation but not at 6-hr incubation in PCLS.

In the assays with PCLS, the accumulation rate of TCBZSO in the culture medium was roughly 30% lower when TCBZ was incubated in the presence of FBZ. Although the intracellular accumulation of both the substrate (TCBZ) and its metabolite (TCBZSO) did not change upon co-incubation, the OFZ-mediated inhibition of BCRP (Merino et al., 2005) may have accounted for the reduced/delayed efflux of TCBZSO. In the end, when assays were carried out in PCLS, both FBZ and OFZ would have been inhibitors of TCBZ metabolism but also of TCBZSO efflux processes.

In both modeling and in vivo studies, combinations have shown to select for resistance at a slower rate than single-dose compounds (Geary et al., 2012). In veterinary medicine, the additional advantages of increased spectrum of activity and efficacy have turned combinations into widely accepted alternatives to single-drug treatments. However, the presence and extent of D-D interactions have not been extensively investigated, leading to a situation in which no safe assumptions can be made as to the real efficacy and clinical effectiveness of co-administered moieties. Accurate predictions of altered pharmacokinetic profiles upon co-administration have become an important concern (Lyubimov, 2011). BDZs are widely used in both human and veterinary medicine, as shown by the ample variety of active principles formulated as diverse pharmaceutical and dosage forms. In vivo studies in female mice have shown synergistic effects for the combination of albendazole and mebendazole against *Trichuris muris* (Keiser, Tritten, Adelfio, & Vargas, 2012), and clinically relevant synergism has also been observed in children infected with *Trichuris trichiuria* (Namwanje, Kabatereine, & Olsen, 2011). The possibility of pharmacokinetic interactions of clinically relevant BZDs for veterinary therapeutics, in particular at the level of metabolism and transport processes, has not been extensively explored and warrants further research. The clinical and practical consequences of this D-D interaction should be evaluated. In this respect, the present study could be complemented with in vivo assays aiming at exploring the clinical and pharmacokinetic effects of sole and concurrent use of FBZ/OFZ and TCBZ in ruminants. Although clinical efficacy of a combined FBZ-TCBZ treatment

has been explored previously (Foreyt, 1988), no comparison between single and concurrent treatments has been established. Such in vivo pharmacokinetic and clinical trials would help to explain whether or not this kind of interaction observed in vitro could result in changes in drug efficacy against target species.

In conclusion, liver slices are an attractive in vitro model to study D-D interactions. They exhibit some promising features compared to other in vitro techniques, such as preserved tissue architecture and cell–matrix interactions. Bovine PCLSs were useful to investigate the effects of the flukicidal compound TCBZ on the metabolic fate of the nematodicidal benzimidazole molecules OFZ and FBZ and vice-versa. Collectively, the effects observed in this study could indicate the existence of more than one level of interaction between molecules. Further in vivo studies are needed to characterize the biological relevance and clinical impact of the described metabolic/transport interactions.

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CONFLICT OF INTERESTS

The authors have no conflict of interests to declare.

AUTHORS' CONTRIBUTIONS

ALL, GLV, and PV conceived the study design and data interpretation. GLV, MLM, and PV carried out the experiments. GLV and PV performed the statistical analysis and interpretation of the data. PV wrote the manuscript with the contribution of GLV, ALL, MLM, and CEL. JPG carried out the histomorphological evaluation. GLV and ALL are involved as project directors. All authors have read and approved the final manuscript.

ORCID

P. Viviani http://orcid.org/0000-0002-7612-0792

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