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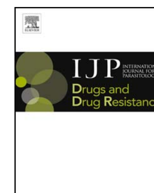

Pathway of oxfendazole from the host into the worm: *Trichuris suis* in pigs

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ABSTRACT

It is well known that the efficacy of a single oral dose of benzimidazoles against *Trichuris* spp. infections in humans and animals is poor, but is currently still used in control programmes against human trichuriasis. However, the route of the benzimidazoles from the treated host to *Trichuris* remains unknown. As parts of adult *Trichuris* are situated intracellularly in the caecum, they might be exposed to anthelmintic drugs in the intestinal content as well as the mucosa. In this study, the pathway of oxfendazole and its metabolites was explored using a *T. suis*-pig infection model, by simultaneously measuring drug concentrations within the worms and the caecal mucosa, caecal tissue, caecal content and plasma of pigs over time after a single oral dose of 5 mg/kg oxfendazole. Additionally, for comparison to the *in vivo* study, drug uptake and metabolism of oxfendazole by *T. suis* was examined after *in vitro* incubation. Oxfendazole and metabolites were quantified by High Performance Liquid Chromatography.

Multivariate linear regression analysis showed a strong and highly significant association between OFZ concentrations within *T. suis* and in plasma, along with a weaker association between OFZ concentrations in caecal tissue/mucosa and *T. suis*, suggesting that oxfendazole reaches *T. suis* after absorption from the gastrointestinal tract and enters the worms by the blood-enterocyte pathway. The fenbendazole sulfone level in *T. suis* was highly affected by the concentrations in plasma. In addition, correlations between drug concentrations in the host compartments, were generally highest for this metabolite. In comparison to oxfendazole, the correlation between plasma and content was particularly high for this metabolite, suggesting a high level of drug movement between these compartments and the possible involvement of the enterohepatic circulation.

1. Introduction

The human whipworm, *Trichuris trichiura*, is one of the major Soil Transmitted Helminths (STHs) and is estimated to infect 465 million people globally (Pullan et al., 2014), primarily in the tropical and subtropical areas of the world (Bethony et al., 2006; de Silva et al., 2003). The current recommendation for control of STHs by the World Health Organisation (WHO) is the use of an oral single-dose treatment with albendazole (ABZ) or mebendazole (MBD) which both belong to the group of benzimidazoles (BZs) (WHO, 2006). However, the efficacy of a single-dose BZ against *Trichuris* spp. infection is medium to low in both humans (Albonico et al., 2003; Keiser and Utzinger, 2008; Knopp et al., 2010; Olsen et al., 2009; Speich et al., 2012; Steinmann et al., 2011) and animals (Batte, 1978; Gordon, 1964; Hebden, 1961).

Similarly, anthelmintics from drug classes other than the BZs, such as ivermectin (IVM) belonging to the macrocyclic lactones (MLs), and levamisole (LEV), an imidazothiazole derivative, have also shown poor cure rates (CRs) against human trichuriasis (Albonico et al., 2003; Belizario et al., 2003; Marti et al., 1996). Also, the amino-acetonitrile derivative drug, monepantel (MOP) has a questionable effect against *Trichuris* spp. in sheep (Sager et al., 2009) and mice (Tritten et al., 2011).

As a consequence of the extensive use of anthelmintics in livestock, resistance to the major drug classes has developed rapidly and now has a large economic impact on livestock production and animal health worldwide (Kotze et al., 2014). Therefore, emergence of anthelmintic resistance in human STHs is of major concern (Vercruysse et al., 2011), particularly with the intended increase of mass drug administration (MDA) programmes initiated by the WHO (WHO, 2012). The naturally low susceptibility of *Trichuris* spp. to a wide range of anthelmintic drugs leaves only few treatment options in MDA control programmes. If resistance to these drugs develop in an already "low susceptible" parasitic nematode, such as *T. trichiura*, the options for future treatments strategies are limited. Indeed, increase in single nucleotide polymorphisms

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(SNPs) associated with BZ resistance, has recently been reported for *T. trichiura* after exposure to ABZ treatment (Diawara et al., 2013). It is therefore of immediate importance to elucidate the reasons why *Trichuris* spp. infections are notoriously difficult to treat in both animal and man when administered as single dose.

Adult *Trichuris* spp. occupy a unique habitat, as the adult worms are situated partly intracellularly in the caecum and the proximal part of colon. The thin anterior part of the worm is embedded in the epithelial layer, whereas the posterior part moves freely in the intestinal lumen (Bundy and Cooper, 1989; Jenkins, 1970; Tilney et al., 2005). Therefore, adult *Trichuris* spp. are potentially exposed to anthelmintic drugs in both the intestinal content and the mucosa.

In a previous *in vivo* study, we observed that in pigs infected with *T. suis* and orally administered the BZ, fenbendazole (FBZ) at a dose range of 5 mg/kg, a positive correlation was evident between drug concentration in pig blood and within the worms of the FBZ metabolite oxfendazole (OFZ), and fenbendazole sulfone ($FBZSO_2$) (Hansen et al., 2014a). These results in pigs suggest that the metabolites enter the worms from the blood-enterocyte pathway rather than directly from the gastrointestinal (GI) tract.

The aim of this study was therefore to explore the pathway of OFZ and metabolites in pigs infected with *T. suis* by determining the drug concentrations in worms, caecal mucosal, caecal tissue, caecal content and plasma, after oral administration of OFZ. For comparison to the *in vivo* study, uptake and metabolism of OFZ and metabolites were determined in worms and culture media after *in vitro* exposure. The *T. suis*-pig infection model was chosen as a model for human trichuriasis, as the pig is considered an appropriate model for drug disposition in humans (Helke and Swindle, 2013) and *T. suis* and *T. trichiura* are closely related species (Hawash et al., 2015; Nissen et al., 2012).

2. Material and methods

2.1. Ethics statement

The study was performed at the Experimental Animal Unit, University of Copenhagen, Denmark according to the national regulations of the Danish Animal Inspectorate (permission no. 2015-15-0201-00760).

2.2. Drugs and chemicals

For the *in vitro* study, FBZ, OFZ and $FBZSO_2$ were purchased from Sigma-Aldrich (Schnelldorf, Germany), and stock solutions of the drugs (10 mM) were prepared in 100% dimethylsulfoxid (DMSO) (Sigma-Aldrich, Schnelldorf, Germany) and stored at 5 °C until use within 1 week. For the *in vivo* study, the commercial formulation of OFZ (Synanthic 9.06%) was purchased from Merial (Lyon, France).

Stock solutions of FBZ, OFZ and $FBZSO_2$ were made in DMSO (12.7 mM) for the analytical work. Mebendazole was purchased from Sigma-Aldrich and used as internal standard. All other chemicals used were of analytical grade.

2.3. Experimental animals and infection

For the *in vitro* study, 4 castrated male pigs (~25 kg) were purchased from a Specific Pathogen Free (SPF) farm (Frenderupgård, Sorø, DK) with no history of helminth infections for 10 years. For the *in vivo* study, 18 castrated male pigs (~25 kg) were purchased from the same farm. On the day of arrival, all animals were weighed, identified by ear tags, and tested for GI parasite infections using the modified McMaster method (Roepstorff et al., 1998). Prior to experimental infection all animals were acclimatized for a period of minimum 1 week. All pigs included in the *in vitro* study, and 15 pigs included in the *in vivo* study were experimentally infected with 5000 embryonated eggs of *T. suis* (kindly provided by Parasite Technologies A/S, Hørsholm, DK) administered through a stomach tube. Patency of infection was confirmed by faecal egg count (EPG) on day 47 and 49 post infection (p.i.) using the modified McMaster technique. The animals had free access to water and were fed restrictively with a diet consisting of 75% barley and 25% supplementary feed (NAG, Helsinge, DK) and were kept at a light/dark cycle of 12 h/12 h. All animals were euthanized by captive bolt pistol followed by exsanguination.

2.4. Experimental design

2.4.1. In vitro study

To compare the accumulation of OFZ and its metabolites $FBZSO_2$ and FBZ within *T. suis*, adult worms were incubated in a final drug concentration of 30 μM in RPMI-1640- medium with DMSO (2% v/v). For each type of drug, 30 worms were incubated at 38 °C (5% CO_2, 21% O_2, 90% relative humidity) for 24 h. This concentration and incubation time were based on previous findings in which maximum specific binding (B_{max}) was reached within 24 h when incubated in a concentration of 10 μM of FBZ (Hansen et al., 2014b). The accumulation of OFZ and $FBZSO_2$ were expected to be lower than FBZ due to their lower lipophilicity, and since previous studies showed that the highest concentration of FBZ soluble at 30 μM, this concentration was chosen (Hansen et al., 2014b). The level of spontaneous drug oxidation in the media, and/or the secretion of drug metabolites from the worms into the media, was determined by measuring the metabolites of the drugs in which the worms were incubated. All incubations were performed in triplicates.

2.4.2. In vivo study

On arrival, the 18 animals were randomly allocated into 5 test groups (group 1–5) and 1 control group with 3 animals in each ($n = 3$). Group 1 and 2, group 3 and 4, and group 5, were infected on 3 consecutive days, respectively. One control pig was infected on each of these days. The pigs were infected on 3 consecutive days due to practical reasons in relation to blood sampling and time of euthanasia. At day 49 p.i. the animals, fasted overnight, were dosed with OFZ by stomach tube at a dose rate of 5 mg/kg. Blood samples were obtained by heparinized Vacutainer tubes (BD Vaccutainer, Kruuse, DK) through vena jugularis 15, 30, 60, 90 min and 2, 3, 4, 6, 8, 12, 24, 32, and 48 h post treatment (p.t.) or until euthanasia. The pigs in group 1, 2, 3, 4, and 5 were euthanized 3, 6, 12, 24 and 48 h p.t., respectively. The selected times of blood sampling and euthanasia were based on a previous pharmacokinetic study of OFZ in pigs (Moreno et al., 2012), and detectable levels of OFZ and $FBZSO_2$ in the caecal content and tissues in pigs from 3 h p.t. with FBZ (5 mg/kg) (Petersen, 1998).

2.5. Recovery of worms, caecal mucosa, caecal tissue and caecal content

2.5.1. In vitro study

Adult *T. suis* were collected from the caecum by manual plucking, and were washed following the washing procedure previously described by Hill et al. (1993). In short, the worms were exposed to 4 consecutive washing steps (each 15 min) in 39 °C Hanks Balanced Salt Solution (HBSS), followed by 4 consecutive washing steps (each 60 min) in 39 °C RPMI-1640 medium. To expose the bacteria to antibiotics already during the washing procedure, both the HBSS and RPMI-1640 medium were supplemented with 1% (v/v) amphotericin B-penicillin-streptomycin solution (10,000 U/mL penicillin, 10,000 mg/mL streptomycin, 25 mg/mL amphotericin B) and 0.5% (v/v) gentamicin (10 mg/mL) (All media, antibiotics and anti-mycotic were purchased from Life Technologies, Naerum, DK).

2.5.2. In vivo study

For the *in vivo* study the pigs were immediately eviscerated and ligatures were placed at the ileum-caecum junction and the caecum-colon junction. The caecum and the large intestine were subsequently

separated from the small intestine.

Caecal content was poured through the caecal-colon opening into a pre-weighed foil tray, weighed, and immediately placed at 105 °C (to avoid potential bacterial reduction of FBZ to OFZ) for 20 h to obtain dry matter. The empty caeca were subsequently opened with scissors and stretched out using needles to fix the tissue.

From each pig, 40 attached worms were collected from the caecal wall. If the number of worms was insufficient, worms from the most proximal part of colon were included. Worms were carefully picked by hand and washed in 50 mL 38 °C isotonic saline for a maximum of 30 s by gently inverting the tube and then poured into a petri dish in approximately 0.5 mL of saline. To remove excess of water, the worms were briefly dabbed on filter paper, using curved forceps, before being transferred into Eppendorf tubes and snap frozen in liquid nitrogen. All samples were stored at −20 °C until high performance liquid chromatography (HPLC) analysis.

Before collecting the whole tissue (i.e. all layers of the caecal wall) and the mucosa, remnants of worms and caecal content were gently removed using a wooden spatula. Whole tissue samples (2 × 2 cm) were cut out at the caecum-colon junction, transferred to 15 mL tubes and snap frozen in liquid nitrogen.

From each pig, a total of 3 g caecal mucosa was gently scraped of the remaining caecal wall using a glass slide. To obtain a representative mucosa sample from the whole caecum, the mucosa was thoroughly mixed in a petri dish before separated into 15 mL tubes (1.5 g in two tubes) and frozen in liquid nitrogen. All tissue and mucosa samples were made in duplicates and stored at −20 °C until HPLC analysis.

2.6. High-performance liquid chromatography analysis (HPLC)

2.6.1. Plasma and worms

Oxfendazole and it's metabolites in worms and plasma were measured using the HPLC method described for plasma (Petersen and Friis, 2000) with minor modifications. Plasma was analysed without internal standard. One mL of plasma was loaded on an activated cartridge (Oasis HLB, 60 mg, 3 mL). The cartridge was activated with 2 mL methanol followed by 2 mL of water. The loaded cartridge was washed with 2 mL 5% methanol and allowed to dry under vacuum for 1 min, before eluting the analyte with 2 mL methanol. The eluate was dried at 37 °C and the residuum was dissolved in 100 μL 50% methanol. This solution was centrifuged at 8000 × *g* before 50 μL were injected into the HPLC-system. Fenbendazole, OFZ and $FBZSO_2$ were diluted in potassium-phosphate-buffer (0.05 M, pH 7.4) at concentrations of 33.4, 31.7 and 30.2 μM from stock solutions in DMSO, work and plasma standards were prepared and run in parallel.

Vials with worms were thawed and then dried under phosphorous pentoxide until constant weight. Each vial with dried worm (29.3–70.2 mg, equivalent to 20–40 worms) was mixed with 200 μL 0.05 M phosphate buffer (pH 7.4) and 200 μL mebendazole (8.5 μM, internal standard) in 0.05 M phosphate buffer (pH 7,4). After gentle homogenization with plastic pestle, 400 μL 6 M guanidine HCl was added. The sample was vortexed for 1 min and placed at 20 °C for 15 min before centrifugation at 8000 × *g* for 10 min. The supernatant was transferred to a clean tube and an additional 400 μL of 6 M guanidine HCl was added to the sample residue. The procedure was repeated and the two supernatants were combined and loaded on activated cartridge (Oasis HLB, 60 mg, 3 mL) as described above. Standards in phosphate buffer and guanidine HCl were run in parallel.

2.6.2. Caecal mucosa, caecal tissue and caecal content

The procedure for the extraction of OFZ and metabolites from tissue and mucosa samples were adopted from Wilson et al. (1991). Samples of 0.5 g tissue/mucosa were added 1800 μL 0.05 M phosphate buffer (pH 7.4) and 200 μL phosphate buffer containing mebendazole as internal standard (33.9 μM). The mixture was homogenised in a plastic tube using an Ultra Torrex homogeniser. Four hundred μL of 4 M potassium carbonate and 2 g of sodium sulphate were added and the tube was shaken at 4 °C in 5 min. The analytes were extracted with 3 × 5 mL ethyl acetate. The ethyl acetate layers were pooled and evaporated at 50 °C under at stream of air. The residue was dissolved in 5 mL n-hexane. One mL of ethanol: 0.2 N HCl (2:1) was added and the mixture was shaken. After centrifugation the hexane layer was discarded and the extraction step was repeated with another 5 mL of hexane. To 750 μL of the ethanol acid layer 2 mL of 2% aqueous potassium bicarbonate solution was added. The entire volume was applied to an activated cartridge (Oasis HLB, 60 mg, 3 mL) as described above.

For the analysis of the caecum content, dried samples were weighed in tarred foil trays and grinded. Samples of 100 mg were mixed with 1900 mL of water and 100 μL of phosphate buffer (0.05 M pH 7.4) containing mebendazol (33.9 μM). The extraction procedure was as described above except that the hexane step was omitted. Standard curves of OFZ and metabolites in tissue and content were run in parallel.

The HPLC system (Waters) was equipped with an autosampler, 2 HPLC pumps, and a UV detector set at 294 nm. Separation of analytes was accomplished at 30 °C on a X-bridge C18 (5 μ, 15 cm). The mobile phase consisted of a gradient mixed from acetonitrile and 0.025 M ammonium acetate (pH 7.2) at a flow rate of 1 mL/min. The proportion of acetonitrile was 35% acetonitrile for the first 4 min, progressing linearly to 70% at 4.5 min, held constant at 70% until 10 min and finally reduced to 35% at 11 min for the remaining run time of 18 min. Retention times for OFZ, $FBZSO_2$ and FBZ were 3 min, 4.8 min and 11 min, respectively. Ratio of peak area of an analyte to peak area of internal standard was used to calculate concentration of the analyte. The method for detecting FBZ, OFZ and $FBZSO_2$ in worms, caecal mucosa, caecal tissue and caecal content was validated in-house by evaluating the following parameters: linear range, recovery, matrix effect, precision, selectivity and limit of quantification. The calibration curves for FBZ were linear between 0.1 and 1 μg loaded on the SPE and for OFZ and $FBZSO_2$ between 0.05 and 0.5 μg loaded on SPE. The recovery of FBZ, OFZ and $FBZSO_2$ was determined by comparing the responses of matrix standards in the range from 0.05 to 1 μg loaded on SPE with water standards in the same concentrations. The recovery was 76%, 101% and 75% for FBZ, OFZ and $FBZSO_2$, respectively. No matrix effect could be demonstrated. The intra-assay and inter-assay variability were 10% and 12%, respectively. The limit of quantification (LOQ) was 1.5, 0.5 and 0.8 mg/g dry worm for FBZ, OFZ and $FBZSO_2$, respectively corresponding to 5.3, 1.7 and 2.4 nmol/g dry worm tissue. Corresponding values for all substances were 0.05 μg/g in tissue and 1.0 μg/g in dried content.

2.6.3. Pharmacokinetics

The plasma concentration was fitted to an open one compartment model with absorption. The pharmacokinetic variables: area under the plasma curve versus time (AUC), the concentration maximum (C_{max}), time of C_{max} (T_{max}) and elimination half-life ($T_{½}$) was calculated for OFZ and $FBZSO_2$ using standard equations (Gibaldi and Perrier, 2007).

2.7. Statistical analysis

Drug concentrations of OFZ and $FBZSO_2$ within worms, caecal mucosa, caecal tissue caecal content, media and plasma are presented as arithmetic means (± SD). For the *in vitro* data, the effects of exposure drug (OFZ, $FBZSO_2$ or FBZ) on (1) the total drug uptake, and (2) the proportion of the total drug uptake corresponding to the same treatment drug, were tested using a Kruskal-Wallis rank sum test in R version 3.4.2 (R Core Team, 2017).

Multivariate linear models were used to evaluate the association between worm drug concentrations and drug concentrations in plasma, caecal mucosa, caecal tissue & caecal content. The parent drug compound and metabolites were modelled separately. In addition, since the caecal mucosa is part of the caecal tissue, drug concentrations in the

caecal mucosa are included in the drug concentrations measured in the caecal tissue. Therefore, two sets of multivariate linear models were used: one analysis including explanatory variables of plasma, content and tissue (Model 1), and a second including explanatory variables of plasma, content and mucosa (Model 2), in order to simplify interpretation between the highly correlated variables of caecal tissue and caecal mucosa. Equations for the full models are given below:

Model 1: $W_i = \beta_0 + ß_P P_i + ß_C C_1 + ß_T T_i + ß_{PC} P_iC_i + ß_{PT} P_iT_i + ß_{CT} C_iT_i + \mathcal{E}_i$

Model 2: $W_i = \beta_0 + ß_P P_i + ß_C C_1 + ß_T M_i + ß_{PC} P_iC_i + ß_{PM} P_iM_i + ß_{CM} C_iM_i + \mathcal{E}_i$

Where β denotes the regression coefficient, Ɛ the residuals and *i* the ith observation of drug concentration in: worms (W), plasma (P), caecal content (C), caecal tissue (T) and mucosa (M).

Within the limits of these full models, a forwards stepwise model selection algorithm was used to select the best fitting final models based on Akaike's Information Criterion (AIC; Akaike, 1973). Residuals from the final models were assessed for normality by visual inspection. All models were run in R with stepwise model selection algorithms from the MASS package version 7.3–47 (Venables and Ripley, 2002).

In order to evaluate the drug pathway from the host into *T. suis*, correlations between the host compartments (i.e. plasma, caecal content, caecal tissue and caecal mucosa) were investigated using Spearman's rank correlation coefficient (ρ). Substantial and significant correlation between compartments was interpreted as “drug movement” between compartments, with an assumed direction based on biologically meaningful interpretations of the relative strengths of each set of correlations. For completeness, simple correlations were also investigated between concentrations in the worm and each of the four host compartments using the same method. Pairwise correlation results were visualised using the ggplot2 package version 2.2.1.9000 (Wickham, 2016) in R.

3. Results

3.1. In vitro study

The *in vitro* studies were conducted to test whether OFZ, $FBZSO_2$ and FBZ were metabolized in the worms. Moreover, the absorption “preference” of OFZ in relation to metabolites was evaluated. The uptake of OFZ, $FBZSO_2$ and FBZ, within *T. suis* and the drug concentration in incubation media are given in Table 1. The total drug concentration within *T. suis* was higher when exposed to $FBZSO_2$ (79.3 ± 2.6 nmol/g wet worm tissue) compared to worms exposed to OFZ (25.6 ± 3.3 nmol/g wet worm tissue) and FBZ (33.2 ± 5.7 nmol/g wet worm tissue). In addition, $FBZSO_2$ constituted 98.96% of the total drug concentration in worms when exposed to this metabolite, which was a higher proportion than OFZ (22.6 ± 2.6 nmol/g worm tissue; 88.3% of total) and FBZ (28.6 ± 5.5 nmol/g worm tissue; 86.2% of total) after OFZ and FBZ exposure, respectively. The overall effect of drug was significant both in terms of the total drug concentration ($p = 0.039$) and metabolite proportion ($p = 0.039$). The overall concentrations of the drug metabolites in the worms and media were low (Table 1), showing that both drug metabolism by the worms themselves and spontaneous oxidation of the drug compounds during incubation were relatively minor. Hence, the drug compound measured within the worms was predominantly the compound surrounding the worms *in vitro*. Interestingly, *T. suis* seemed to have an absorption “preference” of $FBZSO_2$ in relation to OFZ and FBZ.

Table 1
In vitro drug concentrations within *Trichuris suis* and media.

Drug in media (30 μM)	Drug	Drug concentration within *T. suis* (mean ± SD) (nmol/g wet worm tissue)	Drug concentration in media (mean ± SD) (nmol/mL)
OFZ	FBZ	1.7 ± 0.5	< LOD
	OFZ	22.6 ± 2.6	–
	$FBZSO_2$	1.3 ± 0.3	0.04 ± 0.002
	Total	25.6 ± 3.3	
$FBZSO_2$	FBZ	0.7 ± 0.8	< LOD
	OFZ	0.2 ± 0.02	0.008 ± 0.003
	$FBZSO_2$	78.5 ± 1.9	–
	Total	79.3 ± 2.6	
FBZ	FBZ	28.6 ± 5.5	–
	OFZ	0.9 ± 0.2	0.008 ± 0.001
	$FBZSO_2$	3.7 ± 0.2	0.08 ± 0.012
	Total	33.2 ± 5.7	

Arithmetic means (± SD, $n = 3$) of oxfendazole (OFZ), fenbendazole sulfone ($FBZSO_2$) and fenbendazole (FBZ), and concentrations within *Trichuris suis* and media after 24 h exposure to OFZ, $FBZSO_2$ or FBZ (30 μM), respectively.

3.2. In vivo study

The concentrations of OFZ and $FBZSO_2$ in *T. suis*, caecal mucosa, caecal tissue, caecal content and plasma are shown in Fig. 1a–b. The concentrations of FBZ in plasma were mostly below quantification level, and examination of the distribution of residuals from the FBZ model indicated lack of normality. Therefore, Fig. 1c shows only the FBZ concentrations in *T. suis*, caecal mucosa, tissue, and content, and results from the multivariate linear models for the FBZ-data are not shown. Furthermore, insufficient numbers of worms were obtained from one animal in group 3 and 4 respectively, hence, the presented worm-data is based on $n = 2$ at 12 and 24 h p.t.

It is noteworthy that the drug concentrations of OFZ within the worms and the plasma of the pigs showed a relatively similar pattern over the entire sampling period, whereas the similarity in pattern between worms, tissue and mucosa was only observed at 24 and 48 h p.t. (Fig. 1a). Also noteworthy is the relatively high peak in OFZ concentration 6 h p.t. in content (66.8 ± 37.1 nmol/g wet content) and mucosa (179.8 ± 45.0 nmol/g wet tissue). This peak was absent in both worms and plasma, which for comparison, was 15.9 ± 0.7 nmol/g wet worm tissue and 4.4 ± 0.4 nmol/mL, respectively (Fig. 1a). A similarity in pattern between $FBZSO_2$ concentration in plasma, tissue and worms was observed during the whole sampling period, whereas the similarity in pattern between worms, mucosa and content only was seen at 12, 24 and 48 h p.t. The $FBZSO_2$ concentrations during the entire sampling period were higher within the worms compared to concentrations in plasma, caecal mucosa tissue, and content. At 24 h p.t. the concentration were in worms 16.6 ± 0.01 (nmol/g wet worm tissue), in plasma 2.5 ± 0.2 (nmol/mL), in caecal mucosa 2.69 ± 0.2 (nmol/g wet mucosa), in caecal tissue 2.5 ± 0.4 (nmol/g wet tissue), and in caecal contents 1.6 ± 0.2 (nmol/g wet content).

The worm concentrations of the two remaining compounds (OFZ and $FBZSO_2$) were analysed using multivariate linear models. For OFZ, stepwise model selection based on AIC yielded a final model including plasma, tissue, and the interaction of plasma and tissue for Model 1, and a final model including plasma, mucosa, and the interaction of plasma and mucosa for Model 2. For $FBZSO_2$, a final model including only plasma was obtained for Model 1 and an identical model was obtained for Model 2. The adjusted R^2 measures how much variation in drug concentration within the worms the model can account for. In the final models R^2 was 0.94 for OFZ and 0.88 for $FBZSO_2$ in Model 1, and 0.95 for OFZ and 0.88 for $FBZSO_2$ in Model 2. The final model results are shown in Table 2.

There was a strong and highly significant ($p < 0.001$) positive association between OFZ concentrations within *T. suis* and in plasma, along with a weaker association between OFZ concentrations in caecal tissue and *T. suis*. There was also a highly significant ($p < 0.001$) negative interaction between plasma and caecal tissue concentrations,

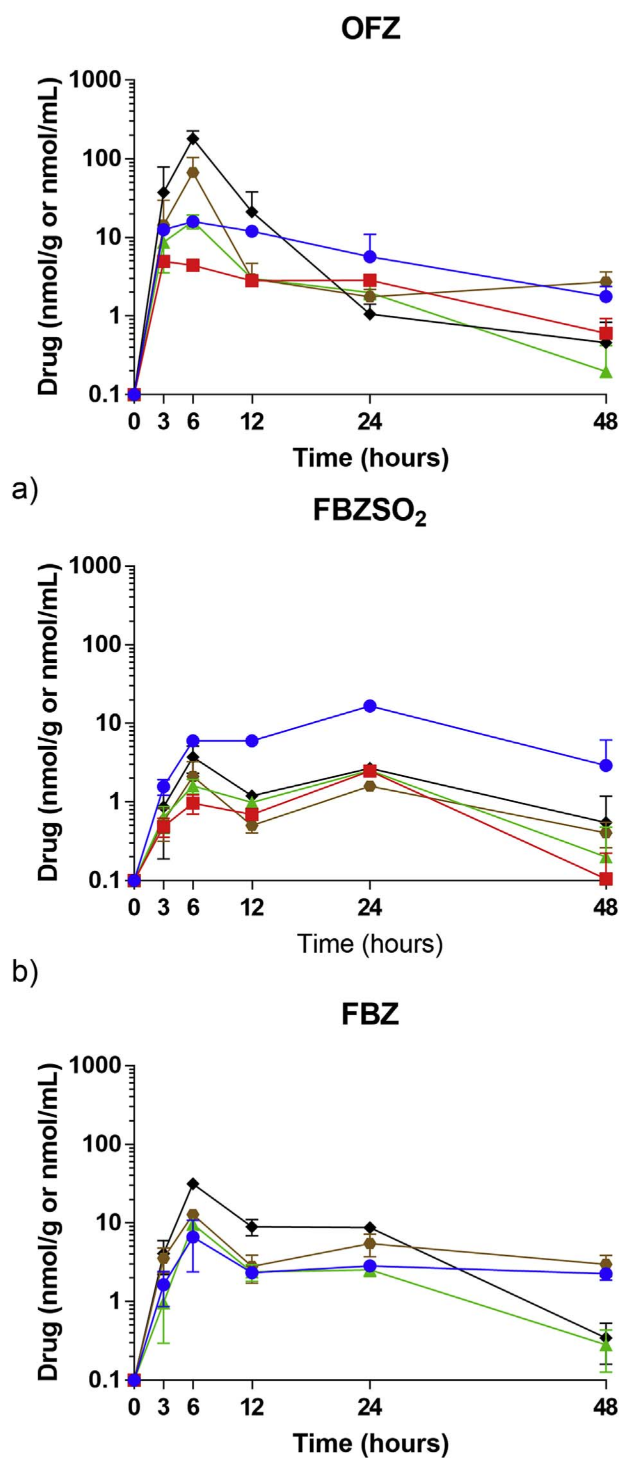

Fig. 1. Mean (± SD) *in vivo* concentrations of a) oxfendazole (OFZ), b) fenbendazole sulfone ($FBZSO_2$) and c) fenbendazole (FBZ) in the worms (●), plasma (■), whole caecal tissue (▲), mucosa (◆) and intestinal caecal content (●) 3, 6, 12, 24 and 48 h after oral administration of OFZ (5 mg/kg). For each point in time $n = 3$, except at 12 and 24 h where $n = 2$.

indicating that an increase in the tissue concentration of OFZ has a smaller effect on the concentration within *T. suis* when the plasma concentration is high (and *vice versa*). Very similar results were obtained for Model 2.

For $FBZSO_2$, a highly significant ($p < 0.001$) positive association was found between concentrations in plasma and *T. suis*, but based on AIC there was no significant effect of either caecal tissue (Model 1) or caecal mucosa (Model 2). Caecal content was not included based on AIC for any of the four models.

Table 2
Regression coefficients (β), standard errors, F values and p values from the final multivariate linear regression models describing the association between drug accumulation within *Trichuris suis in vivo* and drug concentrations in plasma (Models 1 & 2), caecal tissue (Model 1 only) and caecal mucosa (Model 2 only). Note that none of the final models included caecal content as an explanatory variable. The F values and p values presented were obtained from type II analysis-of-variance tables.

	β estimate	Std. error	F value	p value
	MODEL 1: OFZ			
Intercept (β_0)	0.76	3.02	–	–
Plasma (β_P)	8.50	1.19	43.4	< 0.001
Caecal tissue (β_T)	6.73	1.24	8.7	0.012
Plasma x caecal tissue (β_{PT})	−1.22	0.25	23.5	< 0.001
	MODEL 2: OFZ			
Intercept (β_0)	1.47	3.27	–	–
Plasma (β_P)	9.58	1.07	69.9	< 0.001
Caecal mucosa (β_M)	0.59	0.15	11.2	0.009
Plasma x caecal mucosa (β_{PM})	−0.12	0.03	11.5	0.008
	MODEL 1 & 2: $FBZSO_2$			
Intercept (β_0)	2.65	2.64	–	–
Plasma (β_P)	21.16	2.21	91.5	< 0.001

The multivariate modelling results indicate that the strongest association with drug concentration in *T. suis* is found with drug concentration in plasma. However, based on simple univariate analysis, there are significant correlations between the drug concentrations in *T. suis* and each of the four host compartments (plasma is excluded for FBZ) (Supl. Fig. 1). This is due to the correlation between drug concentrations in each of the host compartments (Supl. Fig. 2), which is likely caused by drug movement between these compartments. In particular, $FBZSO_2$ and OFZ appear to be highly correlated between plasma and caecal tissue, and between caecal content and mucosa. In comparison to oxfendazole, the correlations are generally highest for $FBZSO_2$, in particular the correlation between plasma and content.

After oral administration of OFZ (5 mg/kg), OFZ and the metabolite $FBZSO_2$ was detected in plasma as early as 15 min p.t. whereas FBZ mostly were below detection level. The OFZ-data fitted a one compartment model with absorption. The mean (± SD) plasma concentration vs. time profiles of OFZ and $FBZSO_2$ for pig in group 5, which were euthanized 48 h p.t., is shown in Fig. 2. The pharmacokinetic variables of OFZ is presented in Table 3 for pigs in group 5 (mean ±

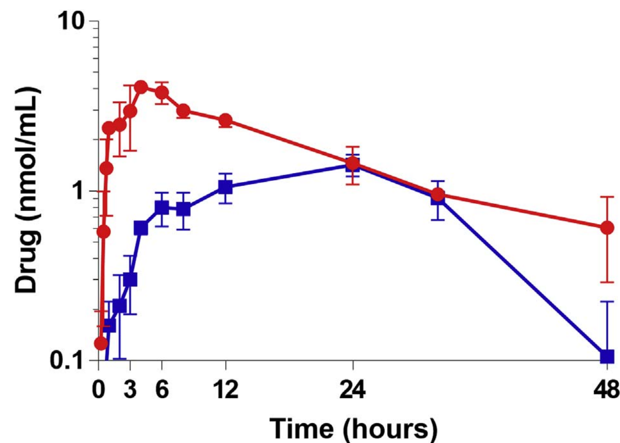

Fig. 2. Oxfendazole (●) (OFZ) and fenbendazole sulfone (■) ($FBZSO_2$) plasma concentrations (mean ± SD) vs. time curve calculated to the last sampling time 48 h p.t. after oral administration of OFZ (5 mg/kg).

Table 3
Plasma pharmacokinetic variables (48 h = group 5; Pooled data = groups 1–5) for oxfendazole (OFZ) in pigs after a single oral dose of Synanthic® 9.06% (5 mg/kg).

Pharmacokinetic variables	48 h (*n* = 3) (mean ± SD)	Pooled data (mean)
$AUC_{(OXF\ 0-C\ last)}$ (nmol h/mL)	82.54 ± 7.96	87.17
C_{max} (nmol/mL)	4.16 ± 0.23	3.77
T_{max} (h)	4.7 ± 0.94	4.0
k_a (h^{-1})	0.55 ± 0.09	0.52
k_e (h^{-1})	0.056 ± 0.002	0.047
$T_{½}$ (h)	12.4 ± 0.5	14.8
F/V (kg/L)	0.31 ± 0.03	0.34
$AUC_{(FBZSO2\ 0-C\ last)}$ (nmol h/mL)	40.12 ± 6.22	44.61
$AUC_{(FBZ\ 0-C\ last)}$ (nmol h/mL)	1.91 ± 1.13	2.26

$AUC_{(0-C\ last)}$: area under plasma drug concentration vs. time curve calculated to the last sampling time 48 h p.t.; C_{max}: peak plasma concentration; T_{max}: time at peak concentration; k_a: absorption constant; k_e: elimination constant; $T_{½}$: elimination half-life; F/V: bioavailability in relation to volume of distribution.

SD) and for all pigs (pooled data, group 1–5).

4. Discussion

We have demonstrated that exposure of *T. suis in vitro* to OFZ, $FBZSO_2$ or FBZ results in accumulation of the same drug compounds in which the worms are incubated, without significant metabolism of drugs in the media or *T. suis*. We therefore presume that the drug concentrations measured within the worms *in vivo*, are related to the drug concentrations surrounding the worms at their predilection site in the caecum. As the thin anterior part of *T. suis* is embedded in the mucosa, and the posterior thicker part is free in the lumen of the caecum, the worms are exposed to anthelmintic drugs from both the caecal content and the mucosa in which they are embedded. In this study, we found that OFZ concentrations within *T. suis* have a significant association with OFZ concentration in plasma, caecal tissue and caecal mucosa. There was no significant effect of caecal content, which might have suggested that OFZ reaching the worms was absorbed from the caecal content. A negative interaction between the effects of drug concentration in the plasma and caecal tissue/mucosa was also found, which indicates that simultaneously increasing concentrations of drug in both plasma and caecal tissue/mucosa result in a smaller increase in concentration within *T. suis* than would be expected from their individual effects. However, the OFZ concentration in plasma seems to have a much stronger effect on the drug concentration within the worms than either the concentration in caecal tissue/mucosa or the interaction between drug concentration within the plasma and tissue/mucosa (Table 2). In addition, the OFZ concentrations in the host compartments, appeared to be highly correlated between plasma and caecal tissue, and between caecal content and mucosa. For the former, we believe that this reflects a particularly high level of drug movement between plasma and tissue. For the later we believe the high correlation should be interpreted with caution, as the caecal content was removed from the mucosa using a wooden spatula, thus, minor faecal material may have been present in the mucosa. Based on our results, we therefore suggest that the major drug pathway of OFZ, after oral administration and absorption across the GI wall of the pig, is through the peripheral bloodstream to the capillaries in the submucosa, from where the drug enter the epithelial layer of the caecum and pass into the worms. A summary of our results, and a suggested pathway of OFZ from the pig host into *T. suis* is depicted in Fig. 3a. The importance of absorbed OFZ and the maintenance of a therapeutic plasma level has previously been shown to have a higher efficacy against *Haemonchus contortus* and *Trichostrongylus colubriformis* in sheep as compared to unabsorbed drug passing down the GI tract (Hennessy and Prichard, 1981). These parasitic nematodes are located in the upper part of the GI tract (i.e. the abomasum and the small intestine) in contrast to *T. suis*, which is located in the hindgut. Our results therefore suggest that OFZ distributed by the blood also has an effect on the drug level in the hindgut of the pig, hence within *T. suis*.

In the multivariate analysis we found that $FBZSO_2$ concentrations within *T. suis* only had a significant association with $FBZSO_2$ concentration in plasma. These results therefore suggest that the major drug pathway of $FBZSO_2$ is through the peripheral bloodstream. However, it is interesting that in the simple univariate analysis, correlations between drug concentrations in the host compartments, were generally highest for $FBZSO_2$, in particular between plasma and content. The sulfone metabolite was detected in plasma as early as 15 min p.t. showing a very rapid sulfoxidation of OFZ to $FBZSO_2$. This sulfoxidation is catalyzed by microsomal enzymes of the cytochrome P450 (CYP) family and the microsomal flavin monooxygenase (MFMO) system (Gottschall et al., 1990) in the liver of mammals, birds and fish (Short et al., 1988), thus, oxidation takes place after absorption from the GI tract. Oxidation of OFZ to $FBZSO_2$ by the bacterial microflora of the intestinal lumen is less likely to occur due to the low redox potential of the intestine which favours reduction (Lanusse and Prichard, 1993). Extensive recycling of FBZ and metabolites (i.e. OFZ and $FBZSO_2$) between plasma and the GI tract via the enterohepatic circulation and reabsorption across the GI tract, has previously been reported in ruminants (Hennessy et al., 1993; Prichard et al., 1981). It is therefore likely that this process also occurs in pigs and that the relatively high correlations between compartments, in particularly between plasma and content, is due to this recycling process, which potentially could expose the worms to $FBZSO_2$ in a continuous manner. A summary of our results, and a suggested pathway of $FBZSO_2$ from the pig host into *T. suis* is depicted in Fig. 3b.

In this study the proportion of adsorbed drugs to the particulate material of the caecal digesta was not determined. As only dissolved drug is bioavailable to the worms (Hennessy, 1993), the drug concentrations exposed to the worms may have been lower than the drug concentrations measured in the caecal content. However, it is possible that the proportion of drugs adsorbed to the particulate material of the digesta, is constant at all sampling times, which in relation to the pattern between drug concentrations in plasma, tissue/mucosa and worms, therefore will show a similar picture (Fig. 1a–b). Most importantly, plasma had the highest F values, hence was the major determinant of the drug levels within the worms. We therefore believe that the relationship between the drug concentrations in the different compartments will be similar to what is described in our study.

The main entry route of BZs into nematodes is reported to be by passive diffusion across the cuticle, and the rate of drug transfer across the cuticle is mainly dependent on the lipophilicity of the drug (Ho et al., 1990, 1992, Mottier et al., 2003, 2006). Hence, it was expected that FBZ, the most lipophilic drug, with an octanol-water partition coefficient of 3.93, would accumulate within *T. suis* at a higher level than OFZ (octanol-water partition coefficient of 2.03) (Mottier et al., 2003) and $FBZSO_2$, which, according to PubChem Substance and Compound database, CID: 162136, has an octanol-water partition coefficient of 2.3, (National Center for Biotechnology Information[1]). It was therefore surprising that the sulfone metabolite, $FBZSO_2$, was the drug compound, which accumulated at the highest concentration within the worms *in vitro*. In fact, the $FBZSO_2$ concentration was more than two fold higher than both FBZ and OFZ. The polar surface area of $FBZSO_2$ is higher (110 A^2) (National Center for Biotechnology Information[1]) than OFZ (103 A^2) (CID: 40854, National Center for Biotechnology Information[2]) and FBZ (92.3 A^2) (CID: 3334, National Center for Biotechnology Information[3]). It is therefore speculated, that the higher polar surface area of $FBZSO_2$, may have an impact on the absorption process. Interestingly, similar *in vitro* results were reported for *T. muris* incubated in ABZ, ABZSO and $ABZSO_2$. In this study by Cowan et al. (2017), $ABZSO_2$ accumulated at a much higher level than ABZ when incubated in 25 μM for 24 h (i.e. 263 ± 173 for ABZ and 421 ± 171 nmol/10 worms for $ABZSO_2$). In the same study, ABZSO

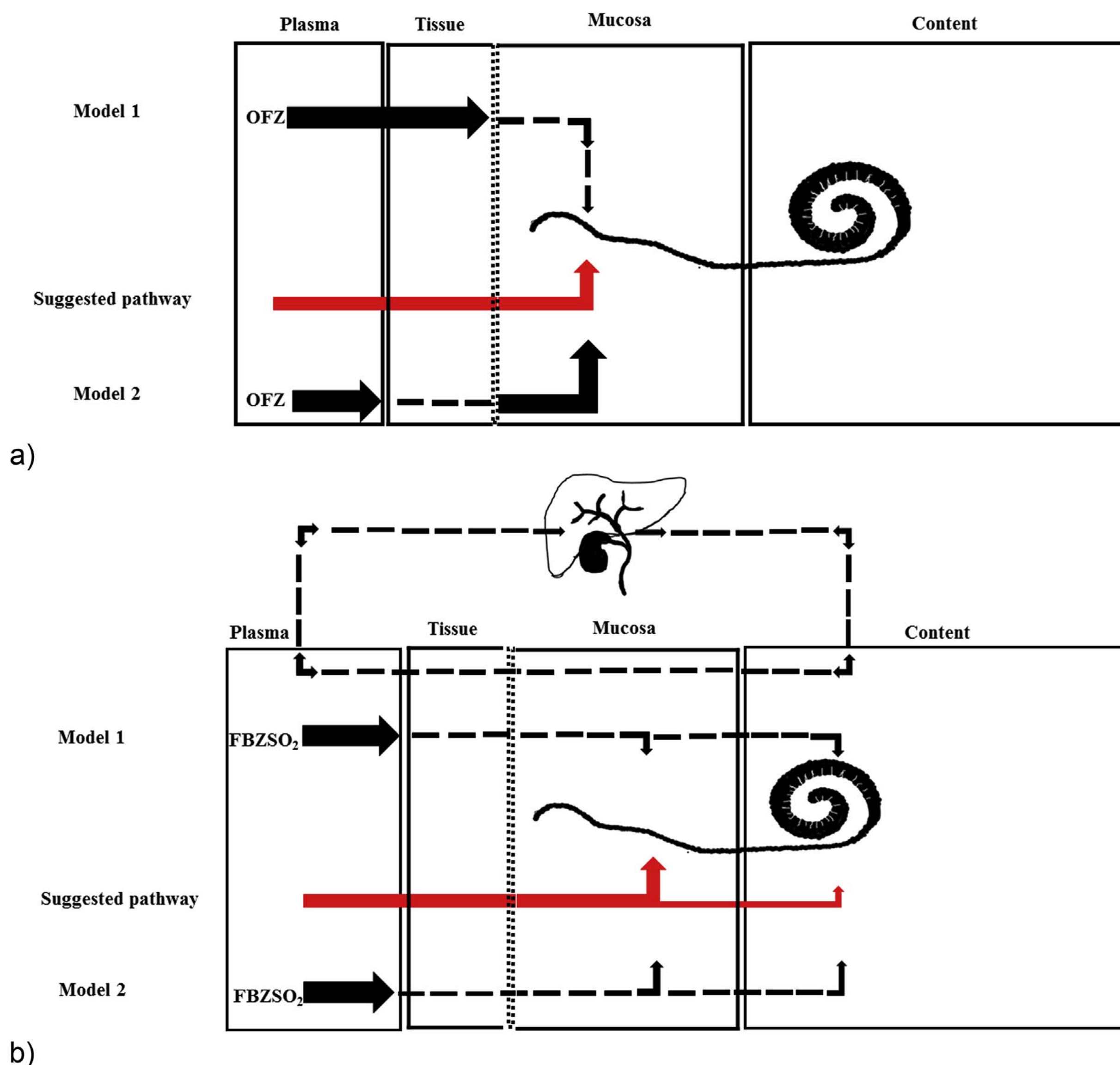

Fig. 3. Scheme of a) oxfendazole (OFZ) and b) fenbendazole sulfone ($FBZSO_2$) distribution in *Trichuris suis* and the compartments: plasma, caecal tissue, caecal mucosa and caecal content of the pig host. Thick black arrows show the compartments involved in drug pathways of OFZ and $FBZSO_2$ from the Model 1 and Model 2 analysis, respectively. Dashed lines indicate assumed drug pathways between compartments based on the simple univariate analysis. Red arrow indicate suggested pathways of OFZ and $FBZSO_2$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

had a tendency of accumulating in *T. muris* at a higher level than ABZ In other helminths, such as the nematode *Ascaris suum* and the trematode *Fasciola hepatica*, ABZSO accumulates at a significantly lower level than ABZ when exposed to ABZ or ABZSO (3.7 µM) *in vitro* for up to 3 h (Alvarez et al., 2001). Similar, when the cestode *Moniezia benedeni* is incubated in FBZ or OFZ (5 µM) for up to 3.5 h, a significantly lower level of OFZ than FBZ accumulates within the worms (Mottier et al., 2003). Whether the high accumulation level of the sulfone metabolite $FBZSO_2$ and $ABZSO_2$ in *T. suis* and *T. muris* respectively, is due to a longer incubation time (24 h) as compared to the studies described above, is yet unknown. However, this study and the study by Cowan et al., show that *Trichuris* spp., accumulate the less lipophilic sulfone metabolites (i.e. $FBZSO_2$ and $ABZSO_2$) at a higher level than the parent compounds (i.e. FBZ and ABZ) when incubated for 24 h.

As previously mentioned, a higher level of $FBZSO_2$ was measured within the worms *in vivo*, as compared to plasma, tissue, mucosa and content. This was not observed for OFZ, although OFZ was the major drug compound in plasma and caecal tissue. Our results therefore suggest that *T. suis*, also *in vivo*, may accumulate $FBZSO_2$ at a relatively higher level than the parent compound OFZ which strongly suggest that an active drug uptake process may take place within the *T. suis*.

The bacillary band of *Trichuris* spp. is situated ventrally on the thin anterior part of the worm which is embedded in the epithelial cells of the caecum and proximal colon (Jenkins, 1970, 1969; Lopes Torres et al., 2013; Lewis G. Tilney et al., 2005). Thus, the bacillary band is in close proximity to the cytosol of the epithelial cells from where nutrient and drugs may be absorbed. Whether anthelmintic drugs are able to enter the worms through the bacillary band is yet unknown, however, previous studies from our laboratory showed that the bacillary band indeed has an absorptive function, at least in relation to glucose (Hansen et al., 2016). It is therefore speculated, that *Trichuris* spp., in contrast to other helminth species, may have an absorption preference of more hydrophilic BZ-metabolites like the sulfones which may enter the worms through the bacillary band by an active uptake process.

We have administered Synanthic® 9.06% at a dose rate of 5 mg/kg body weight. In a previous study by Moreno et al. (2012), a dose of 30 mg/kg body weight was administered to pigs of the Peruvian local ecotypes breed. It appears that in pigs, the plasma AUC is not linear

with this dose since the figure in our study was 26.03 μg h/mL vs 209.9 in the study by Moreno et al. (2012). This difference is mainly ascribed to a faster elimination rate in our study ($T_{½}$ 12.4 h vs. 21.6 h). The metabolism of BZs is catalyzed by CYPs (Gottschall et al., 1990), which expression level is gender age and breed dependent (Helke et al., 2016; Kojima and Degawa, 2016). In our study, the pigs were castrated males, 18 weeks of age, and crossbreeds of Yorkshire, Landrace and Duroc, whereas the pigs in the study of Moreno et al., (2012) were males and females of the Peruvian local ecotypes breed in the age range of 3–6 months. Thus, the faster elimination rate may be due to different experimental conditions such as gender, age and breed. In addition, different types of diet has been shown to affect the plasma pharmacokinetic of BZs in pigs (Alvarez et al., 1996), thus, it is possible, that some of the difference in AUC may be ascribed to the differences in diet.

5. Conclusion

Based on our results, we suggest that OFZ reaches *T. suis* after absorption from the GI tract and is primarily distributed to the worms by the systemic circulation-enterocyte pathway, and that $FBZSO_2$ may reach the worm in a similar manner possibly involving the enterohepatic circulation. In contrast to other parasitic helminths, *T. suis* seems to have an absorption “preference” of less lipophilic, and higher polar surface area BZs metabolites, which may be due to the absorptive function of the bacillary band and its expected function as an external gut. Explorative drug-absorption studies on the bacillary band may therefore reveal important implication of this structure, and increase our understanding of why *Trichuris* spp. in general are less susceptible to a broad range of anthelmintic drugs.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ijpddr.2017.11.002.

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