Trichuris suis and Oesophagostomum dentatum Show Different Sensitivity and Accumulation of Fenbendazole, Albendazole and Levamisole In Vitro

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Abstract

**Background:** The single-dose benzimidazoles used against Trichuris trichiura infections in humans are not satisfactory. Likewise, the benzimidazoles, fenbendazole, has varied efficacy against Trichuris suis whereas Oesophagostomum dentatum is highly sensitive to the drug. The reasons for low treatment efficacy of Trichuris spp. infections are not known.

**Methodology:** We studied the effect of fenbendazole, albendazole and levamisole on the motility of T. suis and O. dentatum and measured concentrations of the parent drug compounds and metabolites of the benzimidazoles within worms in vitro. The motility and concentrations of drug compounds within worms were compared between species and the maximum specific binding capacity (Bmax) of T. suis and O. dentatum towards the benzimidazoles was estimated. Comparisons of drug uptake in living and killed worms were made for both species.

**Principal findings:** The motility of T. suis was generally less decreased than the motility of O. dentatum when incubated in benzimidazoles, but was more decreased in levamisole. The Bmax were significantly lower for T. suis (106.6, and 612.7 pmol/mg dry worm tissue) than O. dentatum (395.2, 958.1 pmol/mg dry worm tissue) when incubated for 72 hours in fenbendazole and albendazole respectively. The total drug concentrations (pmol/mg dry worm tissue) were significantly lower within T. suis than O. dentatum whether killed or alive when incubated in all tested drugs (except in living worms exposed to fenbendazole). Relatively high proportions of the anthelmintic inactive metabolite fenbendazole sulphone was measured within T. suis (6–17.2%) as compared to O. dentatum (0.8–0.9%).

**Conclusion/Significance:** The general lower sensitivity of T. suis towards BZs in vitro seems to be related to a lower drug uptake. Furthermore, the relatively high occurrence of fenbendazole sulphone suggests a higher detoxifying capacity of T. suis as compared to O. dentatum.

Introduction

The whipworm Trichuris trichiura has been estimated to infect 600 million people worldwide resulting in an estimated 1.6–6.4 million disability years lost globally [1]. The current control strategy against T. trichiura and other soil-transmitted helminths (STHs) is administration of single-dose anthelmintic drugs [1,2]. The benzimidazoles (BZs) i.e. albendazole (ALB) and mebendazole (MBD) are widely used in large-scale control programs where they are administered regularly, at a dosage of 400 mg (ALB) or 500 mg (MBD) [2]. However, the efficacy of single-dose BZ against T. trichiura is not satisfactory. A meta-analysis of 20 randomized, placebo-controlled trials reported an average cure rate (CR) of 28% for ALB (400 mg) and 36% for MBD (500 mg) [3]. Other randomized controlled trials have reported similar low CR and egg reduction rates (ERR) ranging from 31.5–40.3% (CR) and 9.8–54.0% (ERR) for ALB and 22.9–66.7% (CR) and 18.8–81.0% (ERR) for MBD [4–7].

The use of the T. musium-mouse model for estimating drug efficacy on T. trichiura is well established [8–11]. Trichuris suis is regarded a different but closely related species to T. trichiura [12,13], hence, T. suis can be considered a valid model for T. trichiura. Another BZ, fenbendazole (FBZ) has shown poor efficacy against T. suis infection in pigs when administered as a single-dose [14], therefore the T. suis-pig model and FBZ may be considered an interesting alternative for studying low treatment efficacy of Trichuris spp. In one controlled trial an oral dose as high as 15 mg/kg, three times the recommended dose of 5 mg/kg for other pig nematodes, was required to obtain a worm count reduction (WCR) of 96.7% [14]. In another controlled study the same oral dose resulted in only a 65.1% reduction in worm burden and a dose of 30 mg/kg resulted in an efficacy of 96.6% [15]. Multiple doses of FBZ (3 mg/kg per day for 3 consecutive days) have shown varied efficacy against T. suis in controlled tests ranging from 66% [16] to 99.8% [14,17] in WCR. The current recommendation...
Author Summary

The human whipworm Trichuris trichiura is together with the roundworm Ascaris lumbricoides and the hookworms Ancylostoma duodenale and Necator americanus the most common intestinal worms worldwide. Together they place more than 5 billion people at risk of infection. The current global control strategy against these worms is regular administration of anthelmintic drugs, mostly albendazole and mebendazole, both belonging to the drug-class benzimidazoles. Both drugs have a low effect against T. trichiura infections, but the reasons for this are not known. We evaluated the in vitro effect of two benzimidazoles; i.e., albendazole, fenbendazole, and another type of anthelmintic, levamisole, on the whipworm (T. suis) and the nodular worm (Oesophagostomum dentatum) of the pig. Oesophagostomum dentatum is highly sensitive towards benzimidazoles in comparison to T. suis. We measured and compared the drug uptake in both species in both living and killed worms. Our results suggest that the reason for the difference in sensitivity is due to a lower drug uptake into T. suis as compared to O. dentatum. Furthermore, T. suis was able to metabolise fenbendazole into an inactive metabolite to a much larger extent than O. dentatum, suggesting a higher detoxifying capacity of T. suis as compared to O. dentatum.

for treatment of T. suis infections in pigs with FBZ is either a single dose of 25 mg/kg, or a long-term treatment where the recommended therapeutic dose is distributed over 7 days [18,19].

Another nematode of the pig is the nodular worm, Oesophagostomum dentatum which in the adult stage, opposed to T. suis, is highly sensitive to FBZ. An oral dose level as low as 0.25 mg/kg has shown an efficacy of 99.9% and doses of 1, 2.5 and 3.5 mg/kg FBZ have resulted in efficacies of 100% in controlled tests based on worm counts [20,21]. Trichuris suis and O. dentatum both inhabit the lower part of the intestine namely the caecum and the colon [22–25], but in their adult stage, their microhabitat varies significantly. The thin anterior part of T. suis is embedded in the mucosa creating a tunnel-like construction of epithelial cells whereas the thicker posterior part of the body is protruding freely into the lumen [26]. In contrast to T. suis, the adult stage of O. dentatum is not attached to the mucosa but roams freely in the intestinal lumen [27,28].

Levamisole (LEV), belonging to another class of anthelmintics, the imidazothiazoles, was introduced in 1968 [29] and has like BZs been used against parasitic infections in both animals and humans. In order for BZs and imidazothiazoles to exert their pharmacological effect, they need to reach their specific receptors within the target parasites i.e. BZs bind to beta-tubulin [30] and the imidazothiazoles to acetylcholine-gated channels [29,31]. Passive diffusion through the external surface has been proposed as the main pathway of BZs (i.e. FBZ, oxendazole (OXF) and triclabendazole sulphoxide (TCBZSO)) in the three main classes of helmint parasites represented by: Moniezia benedeni (cestode), Fasciola hepatica (trematode) and Ascaris suum (nematode) [32]. The uptake of LEV has likewise been demonstrated to occur via a transcellular mechanism in A. suum, but was observed to take place in four distinct stages, thus suggesting a non-passive up-take mechanism [33]. Once inside an organism, drugs are generally being metabolised. However, our knowledge of the metabolism of anthelmintics in helminths is very limited, although drug metabolising enzymes are well described in mammals and serve as an efficient defense mechanism against potential harmful substances. In brief drugs are (if not excreted unchanged) biotransformed by unique enzymes into more polar compounds that are easier to excrete by the organism in metabolic reactions named phase I-III. In mammals the major phase I reaction is oxidation catalysed by cytochrome P450 superfamily (CYPs) [34]. For many years attempts to detect CYPs in parasitic nematodes were unsuccessful [35] but with the discovery of 75 predicted CYP genes in the free-living nematode Caenorhabditis elegans as well as genomic and transcriptomic-based predictions of proteins produced by helminths, the knowledge has improved [36]. The ability of parasitic helminths to metabolise anthelmintics may serve as an advantageous defence mechanism. Previously, the first step of phase I oxidation of ALB into albendazole sulphoxide (ALBSO) (sulphoxidation) has been reported for F. hepatica, M. expansa, A. suum [37], Dicrocoelium dendriticum [38] and Haemonchus contortus [39]. This metabolite has a lower pharmacological activity than the parent compound [40] and lower effect on nematode motility [41]. The second step of ALB oxidation (sulphonation) into alendazole sulphone (ALBSO2) was reported for D. dendriticum [38]. A similar sulphonation process has been reported for F. hepatica exposed to triclabendazole sulphoxide (TCBZSO) in vivo [42]. To the best of our knowledge no studies has been conducted on the metabolism of FBZ within parasitic nematodes. Comparative in vitro studies of the oxidative metabolism of FBZ by hepatic microsomal fractions from a variety of vertebrate species showed that all species readily produced the sulphoxide metabolite (= oxendazole, OXF) and the sulphone metabolite fenbendazole sulphone (FBZSO2) [43]. Oxendazole is a widely used anthelmintic whereas FBZSO2, similar to ALBSO2, are considered pharmacologically inactive [40,44].

We find the different sensitivity of T. suis and O. dentatum to FBZ in vivo highly interesting because these two species are located in the same compartment of the intestine and thus theoretically exposed to similar concentrations of drugs. We speculate that the difference in sensitivity may be related to differences in uptake and/or metabolism of the drug inside the worms. We hypothesized that the reason for a low or variable treatment efficacy of T. suis infections may be due to a lower drug uptake and/or a higher drug metabolism of T. suis in comparison to O. dentatum. The aim of this study was therefore to examine the motility of T. suis and O. dentatum adult worms in vitro when exposed to FBZ, ALB and LEV and to assess whether these drugs accumulate in the same concentrations within the two species.

Materials and Methods

2.1 Drugs

Fenbendazole, ALB and LEV were purchased from Sigma-Aldrich (Schnelldorf, Germany), and stock solutions of the drugs (100,000 μM) were prepared in 100% dimethylsulfoxid (DMSO) (Sigma-Aldrich, Schnelldorf, Germany) and stored at 5°C until use within 1 week.

2.2 Experimental animals and parasite infections

Fourteen pigs were purchased and acclimatized for 1 week prior to experimental infection. The animals had free access to water and were fed restrictively, according to national feeding requirements. For the FBZ in vitro assay, six pigs were orally infected by stomach tube with 2,000 embryonated T. suis eggs
graded as follows: 3: normal motility (movement of the whole body), 2: low motility (slower movement of the whole body), 1: very low motility (movement of the anterior part only), 0: no movements. The motility of *O. dentatum* was graded as follows: 3: normal motility (swimming), 2: low motility (slow swimming or jerking movements), 1: very low motility (only movement of the anterior tip of the body), 0: no movements. All motility measurements were blinded except for worms incubated in FBZ, due to lack of resources.

2.6 Comparison of *in vitro* drug uptake in living and killed nematodes

In order to compare the accumulation of drugs in living and killed worms, a number of worms obtained after the common washing procedure was killed by freezing (liquid nitrogen for 1 min.) and thawed at 5 °C. Thirty living and 30 killed worms of each species were then incubated for 24 hours in FBZ, ALB or LEV at a final concentration of 10 μM in RPMI-1640 medium with DMSO (2% v/v) using the same conditions as described above. All incubations were performed in triplicates.

2.7 Preparation of nematodes and HPLC analysis

After motility measurements and the 24 hour incubation period of living and killed *T. suis* and *O. dentatum*, all worms were carefully rinsed in 50 ml HBSS for a maximum of 30 sec. The *in vitro* assay with FBZ was conducted first, and since the drug concentration within worms was unknown, all worms from each incubation concentration were pooled into one sample to ensure a detectable drug level. Subsequently, triplicates were made for worms incubated in each of the five concentrations of ALB and LEV. After rinsing, worms were transferred to pre-weighed Eppendorf vials, frozen in liquid nitrogen and kept at −20°C until HPLC-analysis.

Vials with worms were thawed and dried under phosphorous pentoxide until constant weight. Each vial with dried worm (10–50 mg) was mixed with 200 μL 0.05M phosphate buffer (pH 7.4) with internal standard (see below). After gentle homogenization with a plastic pestle another 200 μL buffer was added and the homogenization repeated before addition of 400 μL 6M guanidine HCl. The sample was vortexed for 1 minute and left at 20°C for 15 minutes before centrifugation at 8000×g for 10 minutes. The supernatant was transferred to a clean tube and an additional 400 μL of 6M guanidine HCl was added to the sample residue. The procedure was repeated and the two supernatants were pooled and loaded on an activated cartridge (Oasis HLB, 60 mg, 3 mL). The cartridge was activated with 2 mL methanol (100%) followed by 2 mL of water. The loaded cartridge was washed with 2 mL 5% methanol and dried under vacuum for 1 minute, before eluting the analyte with 2 mL methanol. The eluate was evaporated under air at 37°C and the residue was dissolved in 100 μL 50% methanol and centrifuged at 8000×g before 50 μL were injected into the HPLC-system. Standards in phosphate buffer and guanidine HCl were run in parallel. Concentration of analyte in worms was expressed as μg per g dry worm.

The HPLC system was equipped with an autosampler, 2 HPLC pumps, and a UV detector. HPLC conditions for FBZ, ABZ and LEV are described below:

**Fenbendazole.** No internal standard was used in the FBZ analysis. The UV detector was set to 294 nm. Separation of analytes was accomplished at 30°C on a Novapak C18 (5 μ, 15 cm). The mobile phase consisted of a gradient mixed from acetonitrile and 0.025M ammonium acetate (pH 7.2) at a flow rate of 1 mL/min. The proportion of acetonitrile was 30%
acetonitrile for the first 3 minutes, progressing linearly to 40% at 3.5 minutes, held constant at 40% until 11 minutes and finally reduced to 30% at 11.5 min for the remaining run time of 17 minutes. Retention times for FBZ, OXF and FBZSO₂ were 13 min, 2.5 min and 4.5 min, respectively. Standards of FBZ, OXF and FBZSO₂ were prepared from stock solutions in DMSO. Peak area of each analyte was used to calculate concentration. The limit of quantification for FBZ, OXF and FBZSO₂ was 2 ng/mg dry worm.

**Albendazole.** FBZSO₂ was used as internal standard in a concentration of 1 µg/mL. The UV detector was set to 290 nm. Separation of analytes was accomplished at 30°C on a NovaPak C18 (5 µ, 15 cm). The mobile phase consisted of a gradient mixed from acetonitrile and 0.025M ammonium acetate (pH 7.2) at a flow rate of 1 ml/min. The proportion of acetonitrile was 25% acetonitrile for the first 2 minutes, progressing linearly to 50% at 2.5 minutes, held constant at 50% until 9 minutes and finally reduced to 25% at 9.5 min for the remaining run time of 17 minutes. Retention times for ALB, ALBSO, ALBSO₂ and FBZSO₂ (IS) were 9 min, 2 min, 3.5 min and 7 min, respectively. Standards of ALB, ALBSO, ALBSO₂ were prepared from stock solutions in DMSO. Peak high of analyte to internal standard was used to calculate the concentration of analyte. The limit of quantification for ALB, ALBSO and ALBSO₂ was 2, 0.1, 5 ng/g dry worm, respectively.

**Levamisole:** Lidocaine was used as internal standard in a concentration of 5 µg/mL. The UV detector was set to 214 nm. Separation of analyte was accomplished at 30°C on a X-bridge C18 (5 µ, 15 cm). The mobile phase consisted of 25% acetonitrile and 75% phosphoric acid (0.1%) containing 0.1% octansulphone acid at a flow rate of 1 ml/min. Retention times for levamisole and lidocaine (IS) were 6.5 min and 10 min, respectively. Standards of LEV and lidocaine (IS) were prepared from stock solutions in water. Peak high of analyte to internal standard was used to calculate the concentration of analyte. The limit of quantification for LEV was 2 ng/g dry worm.

2.8 Statistical analysis

All motility scores were normalized into percentages relative to controls within species. For each drug the effect of all factors (species, time and log_concentration) and biological meaningful interactions between the factors were tested for statistical significance (P<0.05) using Analysis of Covariance (ANCOVA) with variance heterogeneity using SAS version 9.3 and JMP version 8 (SAS Institute, Cary, North Carolina). Due to significant effects of time, the effect of drug concentrations in the media on the relative motility of the two species was then calculated for 24 and 72 hours separately. Variance heterogeneity was used since the variances between the species were different. Total drug concentrations (parent compound and its metabolites) in living and killed worms of each species were compared using Student’s t-test with variance heterogeneity (JMP version 8). Drug concentrations in worms exposed to 5 µM FBZ resulted in a significant motility decrease for both species time (P<0.0001) (species*time) and the increasing concentration of FBZ resulted in a significant motility decrease for both species time (P=0.012) (log_concentration). When exposed to increasing concentrations of ALB, the motility of **T. suis** was less affected than that of **O. dentatum** after 24 hours incubation in FBZ (P=0.003) but not 72 hours (P=0.73) (species*log_concentration). Although the interaction was not significant for 72 hours, the motility of **T. suis** was significantly less affected than the motility of **O. dentatum** (P<0.0001) (species) and the increasing concentration of FBZ resulted in a significant motility decrease for both species (P=0.012) (log_concentration). When exposed to increasing concentrations of ALB, the motility of **T. suis** was less affected than **O. dentatum** after both 24 hours (P=0.003) and 72 hours (P<0.0001) (species*log_conc). The opposite was observed for increasing concentrations of LEV where the motility of **T. suis** was reduced more than **O. dentatum** after 24 hours (P<0.007) and 72 hours (P<0.007) (species*log_conc).

3.2 Drugs concentrations within living and killed worms

The mean concentrations of the parent compounds FBZ, ALB and LEV and the metabolites of FBZ (OXF, FBZSO₂) and ALB (ALBSO, ALBSO₂) in living and killed worms after incubation in 10 µM of the drug for 24 hours are shown in Fig. 2. In general, the total drug concentrations within both living and killed worm species varied according to type of drug (Fig. 2a, 2b, 2c), with ALB and its metabolite ALBSO occurring at the highest concentration level followed by FBZ and its metabolites and LEV. When incubated in ALB and LEV, the total drug concentrations were found to be significantly lower in **T. suis** than **O. dentatum** and this was observed for both living (ALB: P=0.02, LEV: P=0.02) and killed (ALB: P=0.002, LEV: P=0.008) worms. In both living and dead worms, the total concentration of FBZ and its metabolites was found to be lower in **T. suis** than **O. dentatum**. For the dead worms, the difference was significant (P=0.004) but did not reach significance for living worms (131.1±17.1 pmol/mg dry worm tissue vs. 153.8±33.3 pmol/mg dry worm tissue for **T. suis** and **O. dentatum**, respectively).

For **O. dentatum** the concentration of drug was higher in killed worms as compared to living worms for all three anthelmintics, and the difference was found to be significant when incubated in FBZ (P=0.006) and ALB (P=0.011). For **T. suis** no difference between the living and the killed was observed when incubated in object or organism. NS is the slope of non-specific binding. Background and NS was constrained to 0 since no binding was observed when measuring the negative controls. The difference of K_d and B_max between the species was evaluated on a significance level of α=0.05. Drug concentrations in worms exposed to LEV were compared using Student’s t-test (JMP version 8) because only the two highest concentrations yielded detectable levels within the worms. Thus, concentration difference between and within species was evaluated when worms were exposed to 10 and 200 µM LEV respectively. For each drug, all data sets were tested for normality.

**Results**

3.1 Motility

The relative motility of **T. suis** and **O. dentatum** after exposure to FBZ, ALB and LEV for 24 and 72 hours are presented in Fig. 1. No significant difference in motility between species was observed with increasing concentration over time for FBZ, ALB or LEV (species*time*log_concentration). The motility of **T. suis** was found to be less affected by time (24 vs. 72 h) than **O. dentatum** when exposed to FBZ (P=0.015) and ALB (P<0.0001), but not LEV (species*time). The motility of **T. suis** was significantly less affected than that of **O. dentatum** after 24 hours incubation in FBZ (P=0.003) but not 72 hours (P=0.73) (species*log_concentration). Although the interaction was not significant after 72 hours, the motility of **T. suis** was still significantly less affected than the motility of **O. dentatum** (P<0.0001) (species) and the increasing concentration of FBZ resulted in a significant motility decrease for both species (P=0.012) (log_concentration). When exposed to increasing concentrations of ALB, the motility of **T. suis** was less affected than **O. dentatum** after both 24 hours (P=0.003) and 72 hours (P<0.0001) (species*log_conc). The opposite was observed for increasing concentrations of LEV where the motility of **T. suis** was reduced more than **O. dentatum** after 24 hours (P<0.007) and 72 hours (P<0.007) (species*log_conc).
Figure 1. Mean relative motility (± SD, n = 21) and a tendency line for *Trichuris suis* (dark gray circle) and *Oesophagostomum dentatum* (light gray triangle) after exposure to FBZ, ALB, and LEV for 24 and 72 hours.
doi:10.1371/journal.pntd.0002752.g001
FBZ, whereas the anthelmintic concentration was significantly higher within killed worms when incubated in ALB ($P = 0.009$) and significantly lower when incubated in LEV ($P < 0.001$). The mean concentrations of OXF in living and killed worms, respectively, were found to be 3.4 and 3.5 pmol/mg dry worm tissue for T. suis and 2.6 and 14.4 pmol/mg dry worm tissue for O. dentatum. The pharmacological inactive metabolite FBZSO$_2$ (mean: 12.7 pmol/mg dry worm tissue) was only observed in living T. suis and amounted 9.7% of the total anthelmintic concentration measured within the worms. The mean concentrations of ALBSO in living and killed worms were 93.8 and 71.9 pmol/mg dry worm tissue, respectively, for T. suis and 133.8 and 124.4 pmol/mg dry worm tissue for O. dentatum. Only trace amount of ALBSO$_2$ (4.71 pmol/mg dry worm tissue) were measured in killed O. dentatum.

Figure 2. Mean concentration (± SD, $n = 3$, each replicate consist of 30 worms) of a) FBZ, OXF and FBZSO$_2$, b) ALB, ALBSO and ALBSO$_2$ and c) LEV measured in living and killed Trichuris suis and Oesophagostomum dentatum after incubation for 24 hours in 10 µM of each of the parent compound. Significant difference in total concentration (parent compound+metabolites) between species is indicated with *, significant difference within the species (living and killed) is indicated with △. $P$-values were obtained using Student’s t-test with variance heterogeneity. *$P<0.05$ and **$P<0.01$ and △$P<0.05$ and △△$P<0.01$.

doi:10.1371/journal.pntd.0002752.g002
3.3 Concentrations of total drug within living worms exposed to different drug levels

The concentration of FBZ and ALB inside living *T. suis* and *O. dentatum* after incubation in 0.01, 0.1, 1, 10 and 30 μM FBZ and ALB for 24 and 72 hours is shown in Fig. 3. The $K_d$ and $B_{\text{max}}$ values for each species at 24 and 72 hours are given in Table 1. For both anthelmintic drugs no significant difference in the $K_d$ – values were observed between the species neither after 24 or 72 hours of incubation. The $B_{\text{max}}$ – values were similar for the two species after 24 hours exposure to both BZs, but after 72 hours incubation, these were significantly lower for *T. suis* than *O. dentatum* when exposed to FBZ ($P=0.0001$) and ALB ($P=0.033$).

The concentrations of LEV found within the worms after exposure to 0.01, 0.1, 1, 10 and 200 μM LEV for 24 and 72 hours were only above the detection limit when exposed to the two highest concentrations (Fig. 4). The concentrations of LEV found within the worm species were much lower than ALBSO (Fig. 5). Incubation concentrations below 0.1 μM of FBZ and ALB did not result in detectable levels of metabolites. The concentration of OXF within *T. suis* did not show a concentration or time dependent increase (3.2–5.4 pmol/mg dry worm tissue and 3.8–5.4 pmol/mg dry worm tissue after incubation periods of 24 and 72 hours, respectively) whereas a clear time dependent increase was observed for *O. dentatum* (5.4–7.9 pmol/mg dry worm tissue and 14.2–15.6 pmol/mg dry worm tissue after 24 and 72 hours, respectively). After 24 hours incubation the inactive metabolite FBZSO$_2$ was only detected in *T. suis*. Results were inconsistent.

3.4 Concentrations of drug metabolites in worms exposed to different levels of anthelmintics

The concentrations of the metabolites OXF, FBZSO$_2$ and ALBSO measured within living *T. suis* and *O. dentatum* are given in Fig. 5. The concentrations of OXF and FBZSO$_2$ within the two worm species were much lower than ALBSO (Fig. 5). Incubation concentrations below 0.1 μM of FBZ and ALB did not result in detectable levels of metabolites. The concentration of OXF within *T. suis* thus increased significantly with incubation time ($P<0.0001$) when incubated in 200 μM LEV, whereas the concentration was lower after 72 hours incubation within *O. dentatum* ($P=0.02$).
and are thus not given. After 72 hours incubation, FBZSO₂ was detected within *T. suis* at an incubation concentration as low as 0.1 mM FBZ whereas FBZSO₂ only appeared in *O. dentatum* when incubated in 10 and 30 mM. After 72 hours a concentration-dependent formation of FBZSO₂ (0.9–17.5 pmol/mg dry worm tissue) was measured within *T. suis* where it represented between 6–17.2% of the total drug concentration whereas in *O. dentatum* it only constituted 0.8–0.9%. For both species, the formation of FBZSO₂ appeared to be both time- and concentration-dependent as consistent results only were obtained after 72 hours incubation. The ALBSO metabolite showed a clear tendency to reach a higher concentration within *O. dentatum* than *T. suis* when incubated for both 24 and 72 hours. The formation of ALBSO within the worms appeared to be both time- and concentration-dependent at incubation concentrations ranging from 0.1 μM to 30 μM. Incubation in 30 μM ALB resulted in ALBSO concentrations equal to or below the concentrations formed when incubated in 10 μM. The metabolite ALBSO₂ was not detected within any of the two species. The metabolites OXF and ALBSO showed a clear tendency to reach a higher concentration level within *O. dentatum* than *T. suis* when incubated for both 24 and 72 hours, but in relation to the total drug concentration, the average proportion of the metabolites were approximately the same (OXF: *T. suis*: 4% at 24 hours and 3.6% at 72 hours; *O. dentatum*: 5.6% and 4%, ALBSO: *T. suis*: 11.1% and 13.8%, *O. dentatum*: 15% and 12.2%).

**Discussion**

In the present work, we have combined worm motility with concentration measurements of drug-uptake and drug metabolism in two nematode species that inhabit the same part of the large intestine, but differ significantly in their intestinal microhabitat. Our results show that the motility of *T. suis* was less affected than the motility of *O. dentatum* when exposed to FBZ for 24 hours and ALB for 72 hours, thus indicating a lower sensitivity of *T. suis* as compared to *O. dentatum* towards these compounds. The maximum binding capacity of FBZ and ALB was significantly lower for *T. suis* than *O. dentatum* after 72 hours incubation and the total drug concentrations were significantly lower in living and killed *T. suis*

**Table 1.** Comparison of the binding constant at equilibrium (Kd) and the maximum specific binding capacity (Bmax) of *Trichuris suis* and *Oesophagostomum dentatum* incubated in FBZ and ALB for 24 and 72 hours.

|          | *T. suis* 24 hours | *T. suis* 72 hours | *O. O. dentatum* 24 hours | *O. O. dentatum* 72 hours |
|----------|---------------------|--------------------|---------------------------|---------------------------|
| FBZ      | Kd 0.37             | 0.33               | 1.36                      | 0.54                      |
|          | Bmax 110.9 (**)     | 106.6*             | 147.0                     | 395.2****                 |
| ALB      | Kd 2.03             | 3.57               | 2.87                      | 2.28                      |
|          | Bmax 514.1          | 612.7**            | 513.9                     | 958.1***                  |

Kd is given in μM and Bmax in pmol/mg dry worm tissue. Comparisons are made between nematode species for Kd – and Bmax -values for each time point and P-values indicated for values with same superscript: *P<0.05, **P<0.0001,***.  

Figure 4. Mean concentration of LEV (± SD, n=3) measured in living *Trichuris suis* and *Oesophagostomum dentatum* after incubation in 10 or 200 μM LEV for 24 and 72 hours. Statistically different concentration between species when exposed to either 10 or 200 μM LEV for 24 and 72 hours, respectively are indicated with: *P<0.05, **P<0.01 and ***P<0.001. Statistically different concentration values within the species between 24 and 72 hours are indicated with: *P<0.05 and **P<0.01.

doi:10.1371/journal.pntd.0002752.t001

doi:10.1371/journal.pntd.0002752.g004
as compared to O. dentatum when incubated in ALB. When living and killed worms were incubated in FBZ, only killed T. suis contained a significantly lower drug concentration than O. dentatum. However, collectively these results suggest T. suis to have a lower uptake of FBZ and ALB than O. dentatum. Furthermore, a relatively higher concentration of FBZSO2 was measured in T. suis than O. dentatum, thus suggesting a higher metabolism of FBZ (or OXF) into FBZSO2 in T. suis. Fenbendazole sulphone is considered anthelmintic inactive due to weak ovicidal activity and lack of inhibition of mammalian tubulin polymerization [44]. The equivalent sulphone metabolite of ALB, ALBSO2, has not only shown complete loss of activity in both egg hatch inhibition assays and inhibition of mammalian tubulin polymerization but also decreased binding affinity to nematode tubulin [40]. Whether the latter also applies for FBZSO2 is not known but due to lack of polymerization inhibition, low ovicidal activity and assumed decreased binding affinity to nematode tubulin, FBZSO2 will in the following be considered “inactive”. However, caution must be taken. Due to uncertainty of detection levels within worms in the first trial, triplicates were not made for T. suis and O. dentatum incubated at different drug levels of FBZ (i.e. 0.01–30 μM). Although triplicates were not obtained, concentration agreement was found within the living worms incubated in 10 μM FBZ in the assay of living and killed worms. Furthermore, the formation of FBZSO2 showed a dose dependent formation.

We found that the motility of T. suis as compared to O. dentatum was less affected by increasing concentrations of FBZ and ALB. A low sensitivity to high concentrations of ALB has also been described for T. muris where doses up to 200 μg/ml (equivalent to 754 μM) of ALB were tested against adult and L3 stages of T. muris in vitro [9]. This dose level, which is approximately 25 times higher than the highest concentration used in our study (30 μM) did not reduce the motility of T. muris by 50% (IC50) after an incubation period of 72 hours. In contrast to T. suis, O. dentatum was found to be more sensitive to increasing concentrations of FBZ and ALB when incubated for 24 and 72 hours respectively. The high sensitivity towards increasing concentrations of ALB and FBZ has also been reported by Petersen et al. [41] who found that a concentration of 0.1 μM was able to inhibit migration of O. dentatum through a mesh by 61% for ALB and 69% for FBZ. An increase in concentration to only 3.16 μM increased the inhibition of migration to 75.3% for ALB and 76.2% for FBZ. The high sensitivity towards increasing concentrations of ALB and FBZ has also been reported by Petersen et al. [41], in agreement with our results in vitro, but more importantly, it is also in concordance with the high efficacy of FBZ against O. dentatum reported in vivo [20,21]. Likewise, low sensitivity of T. muris towards ALB in vitro has also been shown to correlate with low treatment efficacy in vivo [9].

Trichuris suis was more sensitive towards increasing concentrations of LEV than O. dentatum. At the highest dose (200 μM) no movement of T. suis was observed neither after 24 or 72 hours incubation. A high sensitivity towards LEV has also been observed for T. muris in vitro (IC50 = 33.1 μg/ml equivalent to 683 μM) and in vivo where the worm burden was reduced by 95.9% with a single oral dose of LEV (200 mg/kg) in mice [9]. In pigs, the efficacy of a single oral dose of LEV (7.5–8 mg/kg) has shown varying efficacy on T. suis ranging from 26% [16] to 100% [48,49].

In the in vitro assay with living and killed worms we found that the total concentrations of anthelmintic drugs were lower in T. suis than O. dentatum (Fig. 2). This applied to all three anthelmintics tested, although the difference was not found to be significant when living parasites were incubated in FBZ (Fig. 2). Incubation in increasing concentrations of FBZ and ALB, ranging from 0.01 to 30 μM for 72 hours revealed similar Ki values for T. suis and O. dentatum which suggests that approximately the same concentrations of FBZ and ALB are needed for both species in order to achieve binding of half of the binding sites at equilibrium. The Bmax values were significantly lower for T. suis than O. dentatum suggesting that T. suis has a significantly lower binding capacity of FBZ and ALB than O. dentatum (Fig. 3, Table 1) which is in accordance with lower effect of these two anthelmintics on motility. The Bmax values measured in O. dentatum were higher after 72 hours than 24 hours incubation. The accumulation of FBZ and ALB may be due to a lower secretion capacity of O. dentatum, in comparison to T. suis, which is supported by the formation of FBZSO2 in T. suis. The concentration of LEV within living worms were below the detection level of the HPLC analysis when incubated in 0.01, 0.1, and 1 μM, but interestingly the concentration of LEV within T. suis was more than two times higher than in O. dentatum when incubated in 200 μM LEV for 72 hours, which was translated into an absence of motor activity in the motility assay.

In the in vitro assay of living and killed worms we found that only living T. suis were able to metabolize FBZ, or possibly OXF, to the inactive metabolite FBZSO2 (Fig. 2), amounting 9.7% of the total anthelmintic concentration measured within the worms. When incubating the worms in increasing concentrations of FBZ for 24 hours we obtained inconsistent results for FBZSO2 (i.e. FBZSO2 was only detected in T. suis, and only when incubated in 1 μM FBZ) (data not shown). After 72 hours a concentration dependent formation of FBZSO2 was measured within T. suis where it represented between 6–17.2% of the total drug concentration whereas in O. dentatum it only constituted 0.8–0.9%. In relation to the maximum binding of FBZ, we measured a significantly lower value for T. suis than O. dentatum (Fig. 3 and Table 1). We therefore suggest that the poor effect of FBZ on T. suis may be related to a lower drug uptake and/or a higher detoxifying capacity of this species, however, some care should be taken with the latter. Albendazole and FBZ are able to undergo spontaneous oxidation to their corresponding derivatives ALBSO and OXF when mixed with DMSO [50]. The average proportions of the metabolites OXF and ALBSO were approximately the same within T. suis and O. dentatum when incubated in increasing concentrations of ALB and FBZ. Furthermore, these metabolites occurred in killed worms of both species and even trace amounts of ALBSO2 were detected in killed O. dentatum. Therefore these findings indicate that OXF and ALBSO were formed by spontaneous oxidation, and that the formation of FBZSO2 observed in T. suis may be related to the presence and further transformation of OXF. As FBZSO2 were not detected in any of the killed worms or in living O. dentatum when incubated in 10 μM FBZ for 24 hours, it is most likely that the relative high concentrations of FBZSO2 measured in T. suis were not formed by spontaneous oxidation, but by T. suis itself. A trace amount of ALBSO2 (4.71 pmol/mg dry worm tissue) was measured in killed O. dentatum when incubated for 24 hours in 10 μM ALB but was not detected in any of the two species when incubated in increasing concentrations of ALB or in dead T. suis. Therefore it is
most likely that occurrence of this compound is a detection uncertainty, which needs to be confirmed in future studies.

The above mentioned findings raise the following questions: a) why is the total drug concentrations of BZs generally lower in *T. suis* than *O. dentatum*? b) Why is the difference between concentration of anthelmintic within living and killed worms more pronounced for *O. dentatum* than *T. suis*? Considering the first question, possible entry routes of anthelmintic drugs into parasitic nematodes are oral ingestion or passive or active transport across the cuticle. In a study performed by Ho et al. [31], transport across the cuticle was demonstrated to be the main route of entry of lipophilic compounds (hydrocortisone and p-nitrophenol) into the nematode *A. suum* [31]. This route was confirmed by Mottier et al. [32], who also suggested that as a general rule helminths uptake BZs by passive diffusion [32]. Since previous work indicated that passive diffusion across the cuticle is the main route of uptake of lipophilic anthelmintics, and a transcuticular route also has been shown for the water soluble anthelmintic LEV [33], we therefore assumed that this also was the case for *T. suis* and *O. dentatum*. Oral ingestion of anthelmintic was controlled in the present study by killing the worms, but the concentration of all three anthelmintics was lower in *T. suis* than *O. dentatum* whether killed or alive, with the exception of living worms exposed to FBZ (Fig. 2). Furthermore, the binding capacity of *T. suis* was significantly lower than the binding capacity of *O. dentatum* when exposed to both FBZ (*P*<0.0001) and ALB (*P*=0.033). The average proportions of the metabolites OXF and ALBSEO were approximately the same for both species, whereas concentration levels above 5 pmol/mg dry tissue of FBZSO₂ were only detected in *T. suis*. We therefore speculate that the lower total drug concentration of BZs measured both in living (i.e. *P*ₘₐₓ, values after 72 hours incubation in ALB and FBZ) and killed *T. suis* may be due to structural differences in the cuticle or different lipid contents. Considering the second question regarding the different concentration of anthelmintic within living and killed worms, Mottier et al. [32] found that the concentration of FBZ was lower within living *A. suum* as compared to killed worms. These findings correspond to our observation for *O. dentatum* exposed to all three anthelmintics, although the difference was not significant when the worms were incubated in LEV (*P*=0.09). For *T. suis*, a significantly lower concentration within living worms in relation to the killed, was only observed when exposed to ALB. The rate of drug diffusion across the cuticle of *A. suum* and other nematodes is restricted by the lipid barrier in the hypodermis, the pKₐ of the drug, the pH of the aqueous environment within the cuticula and the negatively charged aqueous filled pores within the collagen matrix [52]. Mottier et al. [32] suggested that the lower concentration within living worms is related to the acidic environment at the nematode surface that is created by excretion of acidic organic metabolites from the worms [53]. Benzimidazoles are weak bases [54] and may therefore largely exist in their ionized form in the acidic environment at the nematode surface. The ionized form is not readily diffusible through the lipid layer of the cuticle therefore a smaller amount of BZs may enter the living parasites compared to the killed. This mechanism may be the reason why we observed a lower concentration of anthelmintic in living *O. dentatum*, and to a lesser extent in living *T. suis*, compared to the killed specimens.Nevertheless, damage of the cuticle due to freezing and a subsequent increase in permeability or possibly higher drug concentrations trapped in the cuticle of killed worms cannot be ruled out. Furthermore, inactivation of possible ATP-dependent efflux pumps i.e. the ATP-binding cassette (ABC) transporter P-glycoprotein (Pgp) [34,55] may also contribute to the increased drug concentration observed within the killed worms. Interestingly, we did not observe the same for *T. suis* when exposed to FBZ and LEV which further supports our hypothesis that the lower drug concentration measured within this species is also related to a lower drug uptake.

An answer to the intriguing question for low to varied treatment efficacy of *T. trichiura* infections in humans has been sought from a variety of angles. The majority of these has taken an empiric approach by evaluating the effect of different treatment strategies in clinical trials such as: a) comparing the efficacy of single-dose BZs treatment (i.e. ALB (400 mg) and MBD (500 mg)) with the efficacy of combination therapy (i.e. BZs in combination with LEV (40 or 80 mg), ivermectin (200 µg/kg) or diethylcarbamazine (150 mg) [4,5]), b) comparing the efficacy of single-doses with triple-doses of ALB and MBD [6] or c) comparing the efficacy of single and double doses of ALB and MBD given alone or in combination [56]. In the above-mentioned clinical trials the highest CR (70.7%) was obtained using 3×500 mg MBD given over 3 consecutive days [6]. Empiric approaches have also been performed using *T. muris* as a model where the effect of single-drugs (i.e. monepantel, ALB, LEV, pyrantel pamoate and oxantel pamoate) and drug combinations between ALB, LEV, MBD, pyrantel pamoate, oxantel pamoate and ivermectin (IVM) have been assessed in both *in vitro* assays and *in vivo* studies [9,57,58]. Albendazole, given as a single-drug, showed poor effect in *in vitro* (600 mg/kg) and low efficacy in *in vivo* (50–200 µg/ml) [9], whereas the combinations of ALB-MBD, MBD-IVM, MBD-LEV and oxantel pamoate-MBD revealed a strong synergistic effect suggesting combination therapy as a future possibility [57]. Yet other approaches have been used in order to find explanations for low to mediocre treatment efficacy of BZs against *Trichuris* spp. infections. Specific variants of the beta-tubulin gene (i.e. single nucleotide polymorphisms (SNPs) in codon 167, 198 and 200) have been reported to convey BZ-resistance in parasitic nematodes of veterinary importance [59–63] and SNPs in codon 200 have been identified in *T. trichiura* obtained from a human population expected to be unexposed to BZs [64]. Furthermore, there is evidence demonstrating a higher frequency of the resistant genotype in codon 200 (TAC/TAC) in eggs of *T. trichiura* isolated from human populations in Haiti and Kenya after treatment with ALB [65], indicating that anthelmintic resistance may be involved in the low to mediocre treatment efficacy of BZs reported for this genus. However, such SNPs were not found in other *Trichuris* spp. [66], and not systematically in human populations [67].

The present work represents yet another approach to address the intriguing question for low to varied treatment efficacy of *T. trichiura* infections in humans. Based on worm motility, concentration of anthelmintic drugs and their metabolites within the worms and the difference in binding capacity of FBZ and ALB, we suggest that the lower sensitivity of *T. suis* towards these drugs in *in vitro* is, in comparison to *O. dentatum*, due to a lower drug uptake. Furthermore, our data indicate that *T. suis* is able to transform FBZ or OXF into the inactive metabolite FBZSO₂. Whether the drug uptake of *T. suis* in *in vitro* mirrors the drug uptake in *in vivo* is still unresolved. In the host, *Trichuris* spp. are attached to the mucosa with the anterior part which may give the worms a mechanical advantage in relation to anthelmintic treatment (they do not easily get detached even when temporarily deprived for energy or paralysed). Furthermore, such attachment may serve as a protective barrier of the anterior part against active drugs in the intestinal lumen and instead render the worms more exposed to less potent anthelmintic metabolites in the blood. However, the posterior part is largely exposed to drugs in the lumen. We do not know whether the majority of the drug acting on *Trichuris* spp. comes from the intestinal lumen or whether it arrives via the blood
supplying the intestine or both, but by using *T. suis* as a model we have shown that the varied and low drug efficacy against *Trichuris* spp. in animals and humans may be related to low drug-uptake in the worms.

### Acknowledgments

The authors gratefully acknowledge Allan Roepstorff and Christian Kapel from Parasite Technologies A/S, Horsholm, DK for providing *T. suis* eggs. Furthermore, we acknowledge Helena Mejer, Lise-Lotte Christiansen, Gonçalo Pacheco, Gerda Larsen, Anna Sofie Eckhoff and Rikke Jess for technical assistance.

### Author Contributions

Conceived and designed the experiments: TVAH PN SMT CF AO. Performed the experiments: TVAH PN CF. Analyzed the data: TVAH PN CF SMT. Contributed reagents/materials/analysis tools: TVAH CF. Wrote the paper: TVAH CF. Critical comments and suggestions for improved manuscript: PN CF AO SMT.

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