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Research Note

The effects of flubendazole and its metabolites on the larval development of *Haemonchus contortus* (Nematoda: Trichostrongylidae): an *in vitro* study

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Summary

The anthelmintic effects of flubendazole (FLU), its two main metabolites reduced flubendazole (FLU-R) and hydrolyzed flubendazole (FLU-H), and thiabendazole (TBZ) were compared using an *in vitro* larval development test in two isolates of *Haemonchus contortus*, a fully susceptible isolate (HCS) and a multi-resistant isolate (HCR). Results were quantified as 50 % lethal concentration (LC_{50}), 99 % lethal concentration (LC_{99}), efficacy factor (EF), and resistance factor (RF). For HCS, both LC_{50} and LC_{99} of FLU were lower than those of the reference TBZ. The anthelmintic activity of FLU-R in HCS and HCR was 13 and 6 times lower than the activity of FLU, respectively. The anthelmintic activity of FLU-H was negligible (approximately 363 – 853 times lower) compared to that of FLU. Although a marked resistance of the HCR isolate to TBZ was confirmed, only a low tolerance to FLU-R and slightly higher tolerance to FLU were found.

Keywords: benzimidazoles; biotransformation; anthelmintic resistance; larval development test

Introduction

Flubendazole (FLU) is a benzimidazole anthelmintic with a wide spectrum of activity against endoparasites. After administration, FLU has a low bioavailability as more than 50 % of the administered dose is excreted unchanged in the faeces (www.emea.europa.eu/htms/vet/mrls/). An advantage of low levels of gastrointestinal absorption may be the higher amount of anthelmintic agent in the intestinal lumen that remains in contact with the gastrointestinal parasites (roundworms, tapeworms). Helminths, though, can be located in other internal organs, and in such cases the only effective treatment is through absorption and systemic distribution of the active anthelmintic compound into the host's body. FLU, however, is rapidly biotransformed in the gastrointestinal tract and liver: plasmatic concentrations of FLU metabolites prevail over the concentration of the parent drug (www.emea.europa.eu/htms/vet/mrls/). The main biotransforming pathway of FLU involves reduction of the ketone functional group and hydrolysis of the carbamate moiety. Ketoreduction, resulting in reduced FLU (FLU-R), is the major metabolic pathway in chickens, turkeys, and sheep, whereas carbamate hydrolysis, resulting in hydrolyzed FLU (FLU-H), is the major metabolic pathway in pigs (Moreno *et al*. 2004, www.emea.europa.eu/htms/vet/mrls/). Although information on the anthelmintic activity of FLU metabolites may be valuable for the estimation of the complex efficacy of FLU administration to farm animals, no relevant data are available. Moreover, some parasites are also able to biotransform FLU: e.g., FLU-R formation was found in the adult stage of *Haemonchus contortus* (Rudolphi, 1803) (Cvilink *et al*., 2008). The impact of ketoreduction on FLU's anthelmintic properties – impairment or deactivation - has not been studied to date. Addressing these facts, the present *in vitro* study was designed to compare the anthelmintic effects of FLU and its metabolites on two isolates of *H. contortus*, one fully susceptible to benzimidazoles and one multiply resistant to anthelmintics, using a modified *in vitro* larval development test (Várady *et al*., 2007) that is able to distinguish between susceptible and resistant isolates of *H. contortus*. Thiabendazole (TBZ) was used as a reference anthelmintic in this test.

Materials and methods

Chemicals

Flubendazole ([5-(4-fluoro-benzoyl)-1*H*-benzimidazole-2-y1]carbamic acid methyl ester, FLU) and its two main metabolites racemic reduced flubendazole ((±)-[5-(4-

fluorophenyl)hydroxymethyl-1*H*-benzimidazole-2-y1]-carbamic acid methyl ester, (±)-FLU-R) and hydrolyzed flubendazole ([(2-amino-1*H*-benzimidazole-5-yl)-4-fluoro phenyl] methanone, FLU-H) were provided by Janssen Pharmaceutica (Belgium). All other chemicals (HPLC or analytical grade) were obtained from Sigma-Aldrich (Czech Republic).

Parasite isolates

Two isolates of *H. contortus,* one susceptible to anthelmintics (HCS) and one resistant to anthelmintics (HCR), were used in this study. The susceptible isolate HCS was obtained as an inbred isolate of the MHCo1 isolate. This isolate from East Africa is susceptible to all main classes of anthelmintics. The multiresistant HCR was isolated in South Africa (Van Wyk & Malan, 1986). In previous tests, the efficacy of benzimidazole anthelmintics in HCR was 77.4 % (Álvarez- Sánchez *et al*., 2005), and the percentage of resistant alleles defined by molecular tests ranged from 31 % to 33 % (von Samson-Himmelstjerna *et al*., 2009). The efficacy of benzimidazoles against HCS was shown to be 97.8 % (Álvarez- Sánchez *et al*., 2005), and the number of resistant alleles determined by molecular tests was 2 – 6 % (von Samson-Himmelstjerna *et al*., 2009).

Trial design

Prior to the trial, both isolates were routinely maintained by passage through worm-free Merino lambs 5-6 months of age that were housed individually. Lambs were infected orally with 5000 – 6000 L_3 larvae of each isolate. Faecal samples were collected on day 30 and day 35 after inoculation, and nematode eggs for larval development tests were collected by differential sieving through three stacked sieves of 250-, 100-, and 25-µm mesh, successively. The material retained on the 25-µm mesh sieve was washed with water, sedimented, and floated with saturated sodium chloride (Coles *et al*., 1992), followed by washing over a 20-µm mesh sieve with water. The eggs obtained were subsequently used for *in vitro* tests.

In vitro larval development test

The procedure used was a modification by Várady *et al*. (1996) of the technique described earlier (Hubert & Kerboeuf, 1992). The modified method that was used in the current study differs from the method described by Hubert and Kerboeuf (1992) in the use of 96-well microtitre plates. The test was performed in a medium comprising 10 µl drug solution, 110 µl distilled water, 20 µl yeast solution, and 10 µl egg suspension (containing approximately 70 – 100 eggs and amphotericin B at a concentration of 5 $\mu g.ml^{-1}$). The stock yeast solution (30 ml) contained 0.3 g of yeast extract dissolved in 27 ml of 0.85 % NaCl and mixed with 3 ml of concentrated Earle's balanced salt solution. Stock solutions of TBZ, FLU, FLU-H, and racemic FLU-R were prepared by pre-dissolving the drugs in dimethylsulfoxide (DMSO) with subsequent dilution in distilled water (1 : 4). The final concentration of DMSO was 1.3 % in each microtitre well. The final concentrations of TBZ and FLU were 0.0006, 0.00125, 0.0025, 0,005, 0.01, 0.02, 0.04, 0.08, 0.16, 0.32, 0.64, and 1.28 $\mu g.ml^{-1}$. The final concentrations of FLU-R and FLU-H were 0.0065, 0.013, 0.026, 0.052, 0.104, 0.208, 0.416, 0.832, 1.664, 3.328, 6.656, and 13.31 $\mu g.ml^{-1}$. In tests, 96-well microtitre plates were incubated at 27°C. On day 7, the incubation was terminated by addition of 10 µl of Lugol's solution into each well, and the numbers of unhatched eggs and $L_1 – L_3$ larvae in each well were counted under an inverted microscope. The test was performed in two replicates for each drug concentration and was repeated in two independent experiments.

Data analysis

Results of the larval development tests are presented as LC_{50} and LC_{99} values, which are defined as the anthelmintic concentrations where the development of eggs to the L_3 stage is inhibited by 50 % and 99 %, respectively. The data were analyzed using a logistic regression model to determine LC_{50} and LC_{99} (Dobson *et al*., 1987). The LC_{50} gives information on the resistance of the average worm in the population, and LC_{99} shows which proportion of the population is the most resistant. Significant differences ($P \leq 0.05$)

Table 1. Lethal doses (LC; $\mu g.ml^{-1}$; mean ± SD) of thiabendazole (TBZ), flubendazole (FLU), reduced flubendazole (FLU-R), and hydrolyzed flubendazole (FLU-H) for susceptible (HCS) and resistant (HCR) isolates of *Haemonchus contortus* obtained by *in vitro* larval development test

Drug	HCS		HCR	
	LC_{50}	LC_{99}	LC_{50}	LC_{99}
TBZ	0.0051 ± 0.0005	0.0120 ± 0.0007	0.0190 ± 0.0007	1.8448 ± 0.0778
FLU	0.0039 ± 0.0015	0.0100 ± 0.0012	0.0109 ± 0.0009	0.0251± 0.0008
FLU-R	0.0455 ± 0.1511	0.1310 ± 0.0042	0.0637 ± 0.0052	0.1432 ± 0.0127
FLU-H	1.9252 ± 0.1202	3.5251 ± 0.5728	4.5050 ± 0.0990	21.2329 ± 4.3560

between LC_{50} or LC_{99} values obtained for FLU and for the reference TBZ, as well as differences between LC_{50} or LC_{99} values established for FLU and for its metabolites within each isolate, were identified by a one-way analysis of variance followed by a Dunnet's multiple comparison test (Instat, GraphPad Software, San Diego, USA). To express the *in vitro* efficacy of the drugs, the efficacy factor (EF) was used. The degree of anthelmintic resistance was expressed as the resistance factor (RF), calculated as the LC_{50} or LC_{99} value for the resistant isolate (HCR) divided by the respective value for the susceptible isolate (HCS).

Table 2. Efficacy factors (EF) obtained for susceptible (HCS) and resistant (HCR) isolates of *Haemonchus contortus* (abbreviations and methods are the same as in Table 1)

Drug	EF_{50}[a]		EF_{99}[a]	
	HCS	HCR	HCS	HCR
FLU-H / FLU	550.0	430.5	363.4	852.6
FLU-R / FLU	13.1	6.0	13.5	5.7
TBZ / FLU	1.3	1.2	1.7	73.8

[a] LC_{50}/LC_{99} for HCS/HCR obtained with FLU-R , FLU-H, or TBZ divided by LC_{50}/LC_{99} for HCS/HCR obtained with FLU

Results and discussion

The *in vitro* larval development test in this study was chosen due to its sensitivity, reproducibility, and ease of interpretation (Várady *et al*., 2007). Table 1 shows LC_{50} and LC_{99} values of TBZ, FLU, FLU-H, and FLU-R inhibition of larval development of HCS and HCR. In HCS, both LC_{50} and LC_{99} values of FLU were lower than those of TBZ, but the differences were not significant. A comparison of LC_{50} or LC_{99} values of FLU with the LC_{50} or LC_{99} values of its metabolites demonstrated significant differences between FLU and FLU-H ($P < 0.01$). For comparison of FLU and its metabolites, efficacy factor (EF) values were calculated. The results (Table 2) show a negligible efficacy of FLU-H, which was approximately 360 – 852 times lower than the efficacy of FLU. In the case of FLU-R, an efficacy 6- and 13-times lower than the efficacy of FLU was found in HCR and HCS, respectively. Based on these results, FLU-H appears to be an inactive product of FLU biotransformation. The reduction of FLU can also be considered a deactivation reaction, but the deactivation is not complete as partial anthelmintic activity of FLU-R is retained. The residual anthelmintic efficacy of FLU-R may contribute to the total anthelmintic effect of FLU administration, especially in birds and sheep where the plasmatic concentration of FLU-R is more than 10-fold higher than the FLU concentration (Moreno *et al*., 2004; Křížová *et al*., 2009). Another anthelmintic drug, mebendazole, is a chemical relative of FLU. Mebendazole and FLU differ only in one fluorine atom and they undergo the same biotransformation pathways – carbonyl reduction and carbamate hydrolysis (www.emea.europa.eu/htms/vet/mrls/). In contrast to FLU, no anthelmintic activity was found for either metabolite of mebendazole (Meuldermans *et al*., 1976).This fact demonstrates a strong dependence of anthelmintic activity on the chemical structure of drugs.

The anthelmintic efficacies of TBZ, FLU, FLU-R, and FLU-H in HCS and HCR were compared using resistance factor (RF) values (Table 3). The distinction between HCS and HCR isolates is more evident in RF_{99} than in RF_{50}. As shown earlier (Várady *et. al*., 2007), calculation of LC_{99} values can significantly increase the sensitivity of the test and identify resistance even if only a small proportion of the worm population is resistant (high values of RF > 1000 are commonly obtained). However, in this study the LC_{50} criterion is probably more suitable because the estimated values of LC_{99} are sometimes within the concentration range used in the test. Additionally, the main goal of our study was to compare the *in vitro* effect of different benzimidazole anthelmintics and their metabolites, so the comparison of small proportions of a resistant population may not be appropriate. Consequently, resistance factors obtained from the LC_{50} values are more reliable. While we confirmed a marked resistance of HCR to TBZ (RF_{99} = 153.7), only a low tolerance to FLU (RF_{99} =2.6) and very

Table 3. Resistance factors (RF) of susceptible (HCS) and resistant (HCR) isolates of *Haemonchus contortus* (abbreviations and methods are the same as in Table 1)

	RF_{50}[a]	RF_{99}[b]
TBZ	3.73	153.73
FLU	2.79	2.51
FLU-R	1.40	1.09
FLU-H	2.34	6.02

[a] LC_{50} for HCR divided by LC_{50} for HCS
[b] LC_{99} for HCR divided by LC_{99} for HCS

low tolerance to FLU-R (RF_{99} = 1.1) was detected. This finding indicates that TBZ, FLU, and FLU-R may have different mechanisms of action and/or different behaviors (transport, metabolism) in the parasitic organism. We suggest that FLU can be more effective than the other benzimidazoles in the treatment of resistant strains of *H. contortus*. However, it is important to bear in mind that third-stage larvae, which were used in our study, are not the main target of anthelmintic treatments. Therefore, verification of our results by *in vivo* testing is necessary.

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