***In Vitro* Pharmacodynamics of Benzimidazole and Tetrahydropyrimidine Derivatives in European Bison Gastro-Intestinal Nematodes**

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ABSTRACT

The present study was aimed to evaluate the efficacy of anthelmintic agents against intestinal nematodes found in European bison. It was performed between October 2016 and May 2017, using Egg Hatch Assay (EHA) and Larval Development Assay (LDA). The parasites were obtained from faecal samples, harvested from bisons in Romania and Sweden. The efficacy of albendazole (ABZ), mebendazole (MBZ) thiabendazole (TBZ) and pyrantel (PYR) was tested. In EHA, the maximum efficacy was observed in MBZ ($EC_{50} = -0.227 \mu\text{g/ml}$), and then TBZ ($EC_{50} = -0.2228$). ABZ had a weaker result, EC_{50} being $0.326 \mu\text{g/ml}$. All tested benzimidazoles registered hatching percentages below 50%, reflecting the lack of parasitic resistance. MIC obtained in the LDA tests were $0.2144 \mu\text{g/ml}$ for TBZ, $0.2792 \mu\text{g/ml}$ for PYR, $0.5429 \mu\text{g/ml}$ for MBZ, while ABZ came last (MIC = $0.8187 \mu\text{g/ml}$). The *in vitro* tests proved the antiparasitic molecules efficacy against bisons nematode population and a limited risk of inducing resistance phenomena.

Keywords: *benzimidazoles, bison, pharmacodynamics, pyrantel*

INTRODUCTION

Parasite invasions are one of the main diagnosed pathologies of European bison (Krzysiak *et al.*, 2015). If a poorly effective drug is used or if the efficient medication is underdosed, development of drug resistance occurs (Cernea *et al.*, 2015, Woodbury *et al.*, 2011). In order to be able to control these pathologies, it is important to monitor the dewormings and assess the effectiveness of the active ingredients used as antiparasitic medication (Cernea *et al.*, 2005, Demiaszkiewicz *et al.*, 2010, Krzysiak *et al.*, 2015). In this context, the present paper is aimed to evaluate the efficacy of benzimidazole and tetrahydropyrimidine anthelmintic agents against the intestinal nematodes of the European bison (*Bison bonasus*).

MATERIALS AND METHODS

An *in vitro* study was conducted to assess the efficacy of four anthelmintic drugs against in digestive strongylidosis in bison between October 2016 and May 2017. The ovicidal and larvicidal effects of benzimidazole derivatives, albendazole (ABZ), mebendazole (MBZ) and thiabendazole (TBZ) and larvicidal effect of pyrantel (PYR), a tetrahydropyrimidine derivative were evaluated. The gastrointestinal parasites were obtained from faecal samples, harvested from European bisons living in Slivuț Reservation, Hunedoara County and Armeniș Reservation Caraș-Severin County, Romania, as well as Avesta Visentpark, Sweden. The bisons have not been dewormed prior to the collection of stool samples. *Strongyle* eggs were collected by flotation methods and two types of

tests were performed: Egg Hatch Assay (EHA) and Larval Development Assay (LDA). The techniques were performed within the Pharmacovigilance Laboratory of Cluj-Napoca Veterinary Medicine Faculty as per protocol of Le Jambre (1978).

The total number of eggs per milliliter was determined applying the formula:

$$\text{Eggs/ml} = (1000 \times \text{no. eggs per } 50\mu\text{l}) / 50$$

Interpretation of EHA results was obtained by comparing them to the results obtained at a reference concentration of 0.15 mcg/ml, as *strongyle* populations in which eggs hatch in a percent of 50 or above are normally considered resistant. As initial solutions, serial dilutions from 500 mcg to 0.98 mcg active ingredient/ml were used. 2 ml well plates were used for conducting *in vitro* bioassays. In each of the first 10 wells, 790 μl of distilled water, 200 μl of egg suspension and 10 μl of each diluted drug solutions were placed, obtaining the final solutions, from 5.0 to 0.0098 mcg active ingredient/ml. Two controls were used, M_1 control consisted of 200 μl egg suspension and 800 μl distilled water and a the second one, M_2 , consisting of 790 μl distilled water, 200 μl egg suspension and 10 μl of HCl solution. After 24 hours in the thermostat at 27°C, samples were assessed by counting both the *embryonated* and *unembryonated* eggs as well as the hatched larvae.

The egg hatching percentage was determined applying the formula:

$$\text{Egg hatching \%} = [(EE + L_1) / (UE + EE + L_1)] \times 100$$

where EE represents the *embryonated* eggs, L_1 represents larvae L_1 and UE stands for *unembryonated* eggs (Coles *et al.*, 1992, Le Jambre *et al.*, 1978).

For Larval development assay (LDA), we tested the MIC after keeping the exogenous parasitic forms in contact with the antiparasitic medication for 14 days, at a constant temperature of 27°C. Larval development percentage was calculated applying the formula:

$$\text{Larval development \%} = [(L_3 / (UE + L_3))] \times 100$$

where L_3 represents the number of L_3 larvae and UE stands for *unembryonated* eggs.

The results obtained were statistically interpreted using a PC software especially created for this type of calculations, called Anthelmintic Resistance Program (ARP™), obtaining and correlating the following parameters – egg hatching percentage, larval development percentage, regression line and its equation (Y parameters), the mean concentration that inhibited hatching in 50% of the eggs (EC_{50}) and minimum inhibitory concentration (MIC) (Cernea *et al.*, 2010, Coles *et al.*, 1992).

RESULTS AND DISCUSSIONS

In EHA, for TBZ active ingredient, a substantial reduction of first stage larvae was observed, compared to the number of eggs, with 14500 eggs, respectively 4500 L_1 larvae. These were identified only in the case of 0.3125 mcg/ml dilution and in the control samples. The low hatching percentage determined, through statistical analysis, led to the calculation of a negative EC_{50} , i.e. – 0.2228 mcg/ml. To be able to get pharmacologically interpretable data with positive values of EC_{50} , serial dilutions lower than 0.0098 mcg/ml should have been used. At the reference concentration (0.15 mcg/ml), the hatching percentage was null by direct determination and, by mathematical analysis, we obtained a value of - 8.7858 (Tab. 1).

For this molecule, the regression line had a negative trend, with an Y_{max} of - 773.34 mcg/ml, proving that the isolated bison parasite populations only possess a limited capacity to adapt to TBZ based medication.

EHA tests on albendazole showed different hatching percentages depending on the concentrations used. Twenty-four hours after incubation, the ratio between the total number of eggs and the total number of L_1 larvae, was 23500/6500.

Hatching percentage at the reference concentration was 33.33%, below the maximum allowed (50%). Mathematical analysis of the ABZ data showed a EC_{50} of 0.3261 mcg/ml, the hatching percentage at the reference concentration being 33.81 mcg/ml (Tab. 1).

High values were also recorded at the parameters „a” and „b” which determined positive values for Y parameter, with a maximum of 479.19 mc/ml. As a result, the *strongyle* population has a high capacity to adapt in the future to ABZ-based anthelmintic products.

Tab. 1. Egg hatch assay results for thiabendazole and albendazole

Sample	A.i. conc. (mcg/ml)	TBZ				ABZ			
		No. of eggs	L1	Egg hatching %	Regression line equation	No. of eggs	L1	Egg hatching %	Regression line equation
1	5.0000	1000	0	0.00	-773.34	1000	1000	50.00	479.19
2	2.5000	1000	0	0.00	-379.24	1000	0	0.00	249.62
3	1.2500	1000	0	0.00	-182.19	1000	0	0.00	134.83
4	0.6250	1000	0	0.00	-83.67	4000	0	0.00	77.43
5	0.3125	1000	4000	80.00	-34.40	5000	1000	16.67	48.74
6	0.1563	2000	0	0.00	-9.78	2000	1000	33.33	34.39
7	0.0781	1000	0	0.00	2.55	2000	1000	33.33	27.21
8	0.0391	2000	0	0.00	8.70	1000	1000	50.00	23.63
9	0.0195	3000	0	0.00	11.79	3000	1000	25.00	21.83
10	0.0098	1000	0	0.00	13.32	1000	0	0.00	20.94
HCl + H ₂ O	0.0000	500	500	50.00	14.86	2500	500	16.67	20.04
Total		14500	4500			23500	6500		
Parameters		Hatching % ref.		EC ₅₀ (mcg/ml)	Parameters		Hatching % ref.		EC ₅₀ (mcg/ml)
a		-157.64		-0.2228	a		91.83		0.3261
b		14.86			b		20.04		

Note: A.i. conc. = active ingredient concentration, EC₅₀ = mean concentrations that inhibited hatching in 50% of the eggs, Hatching % ref. = Hatching percentage at reference concentration of 0.15 mcg/ml

Tab. 2. Egg hatch assay results for mebendazole

Sample	A.i. conc. (mcg/ml)	MBZ			
		No. of eggs	L1	Egg hatching %	Regression line equation
1	5.0000	1000	0	0.00	-860.66
2	2.5000	4000	0	0.00	-425.19
3	1.2500	1000	0	0.00	-207.45
4	0.6250	1000	0	0.00	-98.58
5	0.3125	5000	0	0.00	-44.14
6	0.1563	1000	0	0.00	-16.94
7	0.0781	2000	0	0.00	-3.31
8	0.0391	1000	100	9.09	3.48
9	0.0195	1000	0	0.00	6.89
10	0.0098	1000	0	0.00	8.58
HCl + H ₂ O	0.0000	1000	1000	50.00	10.29
Total		19000	1100		
Parameters		EC ₅₀ (mcg/ml)		Hatching % ref.	
a		-174.19		-15.8300	
b		10.29			

Note: A.i. conc. = active ingredient concentration, EC₅₀ = mean concentrations that inhibited hatching in 50% of the eggs, Hatching % ref. = Hatching percentage at reference concentration of 0.15 mcg/ml.

It should be noted that in the case of ABZ, *in vivo* results may be very different from those obtained *in vitro*, due to its enterohepatic recirculation, which allows for the appearance of active metabolites that will repeatedly exercise their anthelmintic action. This phenomenon is known to be a drawback of the *in vitro* tests, compared to the *in vivo* faecal egg reduction test (Cerneș *et al.*, 2006). As a result, it can be concluded that for albendazole EHA test results, highlight the ovocidal action.

The MBZ effect on strongyle eggs was highlighted by the fact that, 24 hours after incubation, a very small number of L₁ larvae (1100) was found compared to unhatched eggs (19000). At 0.0391 mcg/ml concentration, a hatching percentage of 9.09% was noticed, while for other dilutions the hatching percentage was null (Tab. 2).

In the control samples, the average hatching percentage was 50% which, by statistical analysis, determined a EC₅₀ of - 0.2270 mcg/ml with a hatching percentage of -5.83%. As with TBZ, in order to obtain positive values for EC₅₀, the serial dilutions should be higher than 5 mcg/ml.

The increased efficacy of MBZ is also proven by the regression line equation, whose Y_{max} parameter was - 860.66, being the lowest value of the tested benzimidazoles. For LDA test, in case of thiabendazole, the ratio between the total egg number + L₁ larvae + L₂ larvae to L₃ larvae, was 11500/7500, showing a different development percentage, depending on the used concentrations. At the maximum concentration of 5 mcg/ml, the development percentage was null, while at 2.5 mcg/ml the percentage was 75.

The maximum larval development percentage was recorded for 0.3125 mcg/ml concentration.

In control samples, average development percentage was 50% and, dependant on this value, mathematical analysis determined a minimum inhibitory concentration (MIC) of 0.2144 mcg/ml. Additionally, the value of the „a” parameter being a negative one (- 148.36), allowed the calculation of the regression line equation, whose maximum Y parameter had the value of -707.48. Therefore, the efficacy of TBZ on this nematode population was also demonstrated by the LDA tests (Tab. 3).

Tab. 3. Larval development assay results for thiabendazole and albendazole

A.i. conc. (mcg/ml)	Sample no.	TBZ				ABZ			
		Eggs + L1 + L2	L3	Larval development %	Regression line equation	Eggs + L1 + L2	L3	Larval development %	Regression line equation
5.0000	1	1000	0	0.00	-707.48	2000	0	0.00	-230.05
2.5000	2	1000	3000	75.00	-336.58	6000	0	0.00	-92.53
1.2500	3	2000	0	0.00	-151.13	3000	0	0.00	-23.76
0.6250	4	1000	1000	50.00	-58.41	1000	0	0.00	10.62
0.3125	5	0	1000	100.00	-12.04	2000	1000	33.33	27.81
0.1563	6	1000	0	0.00	11.13	0	1000	100.00	36.40
0.0781	7	1000	0	0.00	22.73	2000	1000	33.33	40.70
0.0391	8	1000	0	0.00	28.52	3000	0	0.00	42.85
0.0195	9	2000	1000	33.33	31.43	3000	2000	40.00	43.93
0.0098	10	1000	1000	50.00	32.87	0	2000	100.00	44.46
0.0000	H2O +HCl	500	500	50.00	34.32	3000	1000	25.00	45.00
Total		11500	7500			25000	8000		
		MIC - mcg/ml		Parameters		MIC - mcg/ml		Parameters	
		0.2144		a	-148.36	0.8187		a	-55.01
				b	34.32			b	45

Note: A.i. conc. = active ingredient concentration, MIC = Minimum inhibitory concentration

Notwithstanding the fact that *in vitro* testing may not reveal ABZ active metabolites activity and effects, it can be said that results obtained in the LDA test have shown a good efficacy of this benzimidazole.

After 14 days of incubation, the total L₃ larvae number was 8000, compared to the other *exogenous forms*, which were 25000.

The first L₃ larvae identified were observed at 0.3125 mcg/ml dilution, the development percentage being 33%. Maximum rates of development were registered for 0.1563 mcg/ml, and 0.0098 mcg/ml dilutions. Mean development percentage on control samples was 25% and the mathematical calculations highlighted an albendazole MIC of 0.8187 mcg/ml. Regression line tendency was negative, with a value of the maximum Y parameter of -230.05 mcg/ml.

Results obtained by LDA when testing mebendazole showed that total number of infesting larvae was 6500, compared to other *exogenous forms*, which were 17500. At the maximum concentration (5 mcg/ml), development percentage was 33.33%.

The maximum development percentage (100%) was registered for 0.0781 mcg/ml concentration. In control samples, mean development percentage was 50%, determining for MBZ a MIC of 0.5429 mcg/ml. Regression line equation had a downward trend, maximum value of Y parameter being -276.47 mcg/ml (Tab. 4).

In the case of pyrantel (PYR), the larvicidal effect was proven by the fact that null values of larval developmental percentage were obtained at four different concentrations.

Also, a 25% larval development was calculated for 1.25 mcg/ml concentration and 100% larval development for 0.625 mcg/ml and 0.0098 mcg/ml concentrations.

Mean larval development percentage for control samples was 50%. The first stage 3 larvae were identified at the 2.5 mcg/ml concentration, thus allowing, by global mathematical analysis, obtaining of a 0.2792 mcg/ml MIC. For PYR, regression line equation showed the maximum y parameter with the lowest value obtained in the LDA tests, respectively -767.12 mcg/ml. Comparative analysis of the predictive adaptability

Tab. 4. Larval development assay results for mebendazole and pyrantel

A.i. conc. (mcg/ml)	Sample no.	MBZ				PYR			
		Eggs + L1 + L2	L3	Larval development %	Regression line equation	Eggs + L1 + L2	L3	Larval development %	Regression line equation
5.0000	1	2000	1000	33.33	-276.47	2000	0	0.00	-767.12
2.5000	2	1000	0	0.00	-118.60	2000	1000	33.33	-359.55
1.2500	3	3000	0	0.00	-39.66	3000	1000	25.00	-155.76
0.6250	4	1000	0	0.00	-0.19	0	2000	100.00	-53.86
0.3125	5	3000	0	0.00	19.55	1000	0	0.00	-2.92
0.1563	6	3000	0	0.00	29.41	2000	0	0.00	22.55
0.0781	7	0	1000	100.00	34.35	1000	1000	50.00	35.30
0.0391	8	1000	0	0.00	36.81	2000	0	0.00	41.66
0.0195	9	1000	2000	66.67	38.05	1000	1000	50.00	44.85
0.0098	10	1000	1000	50.00	38.66	0	2000	100.00	46.43
0.0000	H2O +HCl	1500	1500	50.00	39.28	500	500	50.00	48.03
Total		17500	6500			14500	8500		
		MIC - mcg/ml		Parameters		MIC - mcg/ml		Parameters	
		0.5429		a	-63.15	0.2792		a	-163.03
				b	39.28			b	48.03

Note: A.i. conc. = active ingredient concentration, MIC = Minimum inhibitory concentration

trend for the strongyle populations obtained from bison has highlighted as through EHA revealed a minimal risk for MBZ ($Y_{\max} = -860.66$), followed by TBZ ($Y_{\max} = -773.34$) and ABZ ($Y_{\max} = 479.19$).

LDA tests showed that the minimal risk of inducing resistance to anthelmintic medication was registered for PYR ($Y_{\max} = -767.12$), followed by TBZ, MBZ and ABZ. From the EC_{50} point of view, the maximum efficacy was recorded in MBZ and TBZ, the last being ABZ with a EC_{50} of 0.326 mcg/ml. However, according to the standard interpretation of this test, in which the tested parasite population is considered to be resistant if the hatching percentage at the reference concentration (0.15 mcg/ml) is higher than 50% (Cernea *et al.*, 2015; Coles *et al.*, 1992), all tested benzimidazoles have been shown to present hatching percentages as low as 50%. From this point of view, the best efficacy was recorded with MBZ, followed by TBZ and ABZ. Determination of MIC for the four tested anthelmintic substances, showed that the lower MIC was 0.2144 mcg/ml for TBZ, followed by PYR with 0.2792 mcg/ml, MBZ with 0.5429 mcg/ml, the last being ABZ with 0.8187 mcg/ml. As for the antiparasitic potency, TBZ and PYR had the best results, followed by MBZ and ABZ.

The benzimidazole group efficacy results are similar to the ones obtained in other bison populations (Demiaszkiewicz *et al.*, 2010). The results show that bison strongyle populations are still very sensitive to benzimidazoles and tetrahydropyrimidines, totally different, compared to strongyle populations in horses, which already developed a wide resistance against benzimidazoles, especially against ABZ (Cernea *et al.*, 2015).

CONCLUSIONS

Both *in vitro* tests showed efficacy of the evaluated antiparasitic active ingredients against strongyle population found in European bison as well as a limited risk of inducing resistance phenomena.

It may be advisable to use MBZ-based products ($EC_{50} -0.2270$) as antiparasitic medication against nematodes for this bison population, but subject to the fact that *in vivo* ABZ, due to its active metabolites, has a longer contact duration and efficacy than other benzimidazoles.

All benzimidazoles have been shown to have a hatching percentage much lower than 50%,

reflecting a lack of resistance against this drug class for the tested strongyle population.

Even if tetrahydropyrimidines do not have ovicidal effects, they have shown a high antiparasitic potency, being a very good alternative in case of benzimidazole resistance.

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