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Development of novel valerolactam-benzimidazole hybrids anthelmintic derivatives: Diffusion and biotransformation studies in helminth parasites

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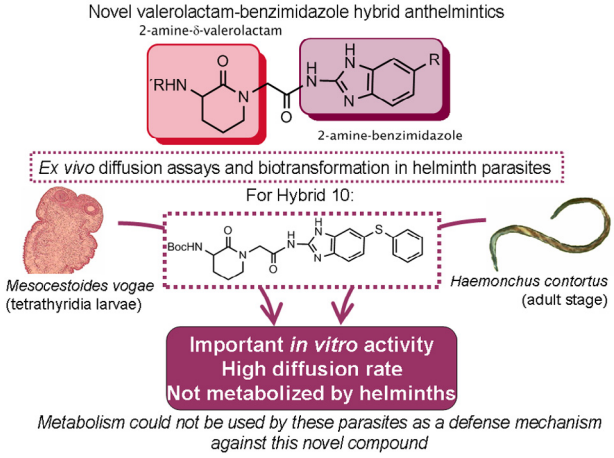
HIGHLIGHTS

- Further studies of novel benzimidazole bioactive hybrids are presented.
- Diffusion in *H. contortus* (susceptible/from sheep farms) and *M. vogae* is presented.
- Sulphoxidation drug metabolism was measured in both target parasites.
- Oxidation was more relevant in *H. contortus* parasites from sheep farms.
- New hybrid compound 10 was not oxidized and showed a high diffusion rate.

GRAPHICAL ABSTRACT



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ABSTRACT

In the search for new anthelmintics able to overcome the resistance problem against all available drugs in livestock, the synthesis of novel valerolactam-benzimidazole hybrid compounds was reported. This allowed us to obtain these *in vitro* and *in vivo* bioactive compounds using *Nippostrongylus brasiliensis* rat model by integrating physiology-based assays and *ex vivo* diffusion studies. In order to further study those novel hybrid molecules, *Haemonchus contortus* (a sheep gastrointestinal nematode of interest) and *Mesocestoides vogae* tetrathyridia (a useful system to study the efficacy of anthelmintic drugs against cestoda) were used as parasite models to compare the *ex vivo* patterns of diffusion and biotransformation of benzimidazoles and their valerolactam-benzimidazole hybrid derivatives. On average, a nine-fold higher intraparasitic concentration of compounds was found in *M. vogae* compared with *H.contortus*, with

Abbreviations: BZ, benzimidazoles; ABZ, albendazole; FLU, flubendazole; FEB, febendazole; ABZ SX, albendazole sulfoxide; FEB SX, febendazole sulfoxide.

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similarities regarding the order of entry of compounds, highlighting febendazole (FEB) and its hybrid compound 10, while valerolactam compound 2 practically did not penetrate the parasites. Interestingly, sulphoxidation drug metabolism was observed and measured, revealing percentages of oxidation of 8.2% and 14.5% for albendazole (ABZ) and febendazole respectively in *M. vogae*, while this effect was more relevant in *H. contortus* parasite. More importantly, significant differences were observed between anthelmintic-susceptible adult parasites (*Hc S*) and those from sheep farms (*Hc U*). In fact, the percentages of oxidation of FEB and the hybrid compound 8 were higher in *Hc U* (25.5%, 54.1%, respectively) than in *Hc S* (8.8%, 38.2%). Interestingly, sulphoxidation of hybrid compound 10 was neither observed in *M. vogae* nor in *H. contortus* parasites, suggesting that increased drug metabolism (oxidation reactions) could not be used by these parasites as a defense mechanism against this novel drug.

1. Introduction

Parasitic helminth infections are a major issue causing serious health and economic problems for livestock worldwide (Neiuwhoff and Bishop, 2005). The Nematoda class, such as *Haemonchus contortus* (one of the most prevalent in sheep), is the single most important constraint to sheep production, causing significant economic losses (Waller, 2006). Since chemotherapy remains the most accessible means to fight helminth parasites, continued heavy reliance on anthelmintic drugs has led to the development of resistance in many helminth isolates. As a consequence of the prevalence of multiple-resistant parasites, it is not uncommon to find sheep farms where animals show resistance to most common available anthelmintic drugs (Skrebsky et al., 2010; Torres-Acosta et al., 2012). In particular, the vast majority of parasites from sheep farms (>80%) in Uruguay were resistant to the main anthelmintic groups used in sheep (benzimidazoles, imidazothiazoles, macrocyclic lactones) (Bonino and Mederos, 2003).

In this context, it is necessary to invest in the search for new anthelmintics with novel biological pathways, which will make it possible to overcome the resistance problem (Geary et al., 2004).

Drug resistance can arise in different ways such as changes of the sites for binding of drugs, detoxifying processes, and increased drug efflux by membrane transporters (James et al., 2009). Although the full mechanism of resistance development has not been thoroughly elucidated yet, it is probable that additional mechanisms of resistance already exist, especially in multi-resistant isolates. Furthermore, an increased drug metabolism produced by the action of xenobiotic metabolizing enzymes is a possible way to facilitate drug resistance. In this regard, enhanced S-oxidative metabolism in triclabendazole-resistant *Fasciola hepatica* was shown (Alvarez et al., 2007). Benzimidazole-2-carbamate (BZ) derivatives are among the most widely used antihelmintic drugs with a broad spectrum of action (including nematode and some cestode helminths) and efficacy, but their intensive and inadequate use has contributed to the development of resistance. In fact, BZ inhibit the microtubule polymerization pathway through binding selectively to the β-tubulin subunit where mutations that led to drug resistance have been identified (von Samson-Himmelstjerna et al., 2009). However, in a recent communication, interest in these molecules was reconsidered. In fact, based on possible tools described at molecular level (docking and dynamics) for BZ derivative optimization, these findings have been suggested as useful to design more potent and selective drugs (Aguayo-Ortiz et al., 2013).

In this context, we have recently reported (Munguía et al., 2013) the design and preparation of hybrid molecules with a dual mode of action (Meunier, 2008) to create efficient new anthelmintic drugs. Novel valerolactam-benzimidazole hybrids were synthesized based on the fusion of two active fragments (Fig. 1). This strategy was used to improve physicochemical properties regarding *in vitro* bioactive valerolactam moiety (compound 2, Munguía et al., 2013), together with the *ex vivo* ability of compounds to diffuse into the target parasite studied (the rat parasitic nematode *Nippostrongylus brasiliensis* four-stage). In that report we have showed the usefulness of diffusion studies jointly with *in vitro* physiology-based assays to search for anthelmintics.

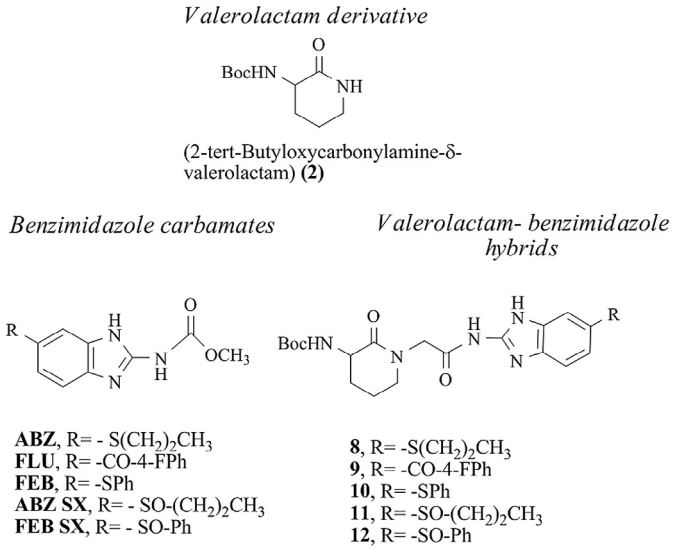

Fig. 1. Chemical structure of compounds.

In this work, *H. contortus*, a sheep gastrointestinal nematode parasite of interest, and *Mesocestoides vogae* tetrathyridia (syn. corti, Cestoda: Cyclophyllidea), a useful system to study the efficacy of anthelmintic drugs against cestodes (Saldaña et al., 2001, 2003), were used as parasite models to compare the *ex vivo* patterns of diffusion of different BZ anthelmintics, their corresponding novel hybrids molecule derivatives, and valerolactam compound 2 (Fig. 1). Also, as increased drug metabolism could be an additional mechanism of resistance, we specifically explored sulphoxidation drug metabolism. We focused on the study of oxidized metabolites produced by means of comparative diffusion assays of those compounds: an anthelmintic-susceptible isolate of *H. contortus* adult parasites (*Hc S*) and those recovered from sheep farms (*Hc U*), which were used under the assumption that they will probably be resistant to BZ anthelmintics (Bonino and Mederos, 2003).

2. Materials and methods

2.1. Chemicals

Albendazole (ABZ), flubendazole (FLU) and febendazole (FEB) were kindly supplied by Laboratorio Uruguay S.A. (LUSA). Phenacetin was used as internal standard (IS) as previously reported (Domínguez et al., 1995). The new series of hybrid compounds valerolactam-benzimidazole (compounds 8, 9 and 10) and valerolactam compound 2 were synthesized according to our recent findings (Munguía et al., 2013). Sulphoxide metabolites of ABZ, FEB, and hybrid compounds 8 and 10 (ABZ SX, FEB SX, 11, and 12, respectively) were synthesized and characterized as described in the Supplementary data. Chemical structures are shown in Fig. 1.

All solvents and other chemicals (HPLC or analytical grade) were provided by J.T. Baker, Xalostoc, Mexico.

2.2. Collection of parasite material

2.2.1. Mesocestoides vogae *tetrathyridia (TT)*

M. vogae isolate maintenance and the recovery of specimens of tetrathyridia (TT) were conducted as we have previously reported (Saldaña et al., 2001, 2003). Briefly, male CD1 mice (2 months old) were infected with fresh *M. vogae* TT in saline solution (0.1 ml, ca 200 TT) by intraperitoneal inoculation. After 2 months the animals were sacrificed by cervical dislocation and the parasites were recovered from the peritoneal cavity, washed twice with PBS (pH 7.4) and maintained for 1 hour at 37 °C and 5% CO_2, until the *ex vivo* experiments were conducted. The animal protocol complied with Uruguayan Law No. 18.611 (http://archivo.presidencia.gub.uy/_web/leyes/2009/10/EC1395.pdf) and it was harmonized with The Canadian Guidelines on Animal Care. Experimental protocol no 02-05-10 of the study was reviewed and approved by IACUC of Facultad de Química – UdelaR, Uruguay.

2.2.2. Haemonchus contortus *adult stage (Hc U, Hc S)*

The *H. contortus* nematodes (third stage, L3) from the anthelmintic-susceptible McMaster isolate were kindly provided by Dr. A. Kotze and Dr. M. Knox (CSIRO McMaster Laboratory, Armidale, NSW). The infection was maintained (infection rate was 10,000 L3 per sheep) at the Campo Experimental de Higiene of Facultad de Medicina, UdelaR. The adult worms (*Hc S*) used in this study were recovered from sheep abomasa (10–15 weeks after infection) by manual picking. The parasites were washed and placed in PBS at 37 °C and 5% CO_2 for 1 hour until *ex vivo* experiments were conducted.

The animal protocol complied with Uruguayan Law No. 18.611. Experimental protocol no 071140-001021-11 of the study was reviewed and approved by IACUC of Facultad de Medicina – UdelaR, Uruguay.

Additionally, adult worms (*Hc U*) recovered from abomasa of naturally infected animals from sheep farms (obtained from a national slaughterhouse, Frigorífico Las Moras, Chiadel S.A., La Paz, Canelones, Uruguay) by manual picking were also used in this study, and were processed as it was described for *Hc S*.

2.3. Ex vivo *drug diffusion assays*

An aliquot of fresh living parasites of *M. vogae* TT (0.4 ml) or fifty female fresh living *H. contortus* adult parasites were incubated in cell culture bottles with 3 or 5 ml of PBS respectively (at 37 °C and 5% CO_2) containing the drug of interest at a final concentration of 5 nmol/ml (five replicates at least) for 30 minutes. Blanks of drug-free sample of parasites were also included. Once incubation time had elapsed, the parasite material was washed twice with 5 ml of PBS, transferred into plastic tubes, frozen and stored at –20 °C until HPLC analysis was conducted.

2.4. Sample preparation and solid-phase extraction

2.4.1. M. vogae *TT*

The parasite material (0.4 ml *M. vogae* TT plus 0.3 ml of distilled water) was quickly homogenized with ultrasound (Cole Parmer CPX 600, Mernon Hills, Illinois, USA) at 0 °C and 23% of amplitude (3 cycles of 1 minute each). Two aliquots of 10 µl were kept for protein determination. The homogenates were spiked with phenacetin (internal standard: IS, final concentration of 22 nmol/ml) and 3 ml of acetonitrile, shaken (2 min, vortex) and centrifuged (15 min, 1000 g). The supernatant was taken and evaporated to dryness. The residue was dissolved in 1 ml of methanol/water (20:80) and injected into Sep-Pak C18 extraction cartridge previously conditioned (by washing with 10 ml of methanol and 10 ml of distilled water). The cartridge was flushed with 10 ml of distilled water and eluted with 3.5 ml of methanol (discarding the first 0.5 ml). It was then evaporated to dryness, and the residue was dissolved with 100 µl of methanol and analyzed by HPLC. Blank samples of drug-free parasites as well as blank samples containing drugs and PBS instead of parasites were similarly processed.

2.4.2. H. contortus *(adults)*

The parasite material (50 specimens with 0.7 ml of phosphate buffer pH 7.8) was homogenized with ultrasound (Sonics Vibra Cell VCX130, Newtown, Connecticut, USA) at 0 °C and 45% of amplitude (8 cycles of 30 s each). Two aliquots of 10 µl were kept for determining protein concentration. A volume of 1.4 ml of acetonitrile was added to the homogenates, stirred for 15 s, and spiked with IS phenacetin (19 nmol/ml final concentration) and 3 ml of acetonitrile. Samples were shaken (2 min, vortex) and centrifuged (15 min, 1000 g). The supernatants were evaporated to dryness and were reconstituted in 1 ml of methanol–phosphate buffer pH 7.8 (20:80), injected into a Sep-Pak C18 extraction cartridge (previously conditioned by washing with 10 ml of methanol and 10 ml of phosphate buffer pH 7.8), and flushed with 10 ml of phosphate buffer pH 7.8, followed by elution with 4 ml of methanol (discarding the first 0.5 ml). The eluate was evaporated to dryness and the residue was dissolved with methanol and analyzed by HPLC. Blank samples of drug-free parasites as well as blank samples containing drugs and phosphate buffer instead of parasites were similarly processed.

The use of phosphate buffer (pH 7.8) instead of water (as in the clean-up and extraction procedure for *M. vogae*) was necessary to improve recovery of compounds from *H. contortus* parasite matrix (see discussion later).

2.5. Parasite protein concentration

Parasite protein concentration was determined according to Smith et al. (1985) with a BCA Protein Assay Kit (Thermo Fischer Scientific, Rockford, IL, USA).

2.6. HPLC analysis and method validation

The liquid chromatography analysis was performed using Waters HPLC equipment, with binary pumps (Waters 1525) and photodiode array detector (Waters 2996), with loop of injection of 20 µl (Rheodyne 1727). A reverse phase C18 separation column was used (Zorbax Eclipse XBD, Agilent, 4.6 mm × 150 mm, 5 µm), with detection at 290 nm for all compounds except for valerolactam derivative 2. A mobile phase with a mixture of ammonium acetate buffer (2 g/l, pH 6.8)–methanol–acetonitrile (60:20:20), at a flow rate of 1 ml/min was used to elute the analytes: ABZ SX, IS, 11, FLU, ABZ, 9 and 8. Initial conditions 60:20:20 were maintained for 5 min, and then changed in 3 min to 42:14:44, maintained during 8 minutes and finally modified to 39:13:48 in 4 minutes. The retention times under these chromatographic conditions were: 3.3, 4.8, 7.9, 8.8, 10.0, 11.2 and 13.4 min for ABZ SX, IS, 11, FLU, ABZ, 9 and 8, respectively. A mobile phase of a mixture of acetic acid 1%–methanol–acetonitrile (70:15:15) at a flow rate of 1 ml/min was used for the analysis of FEB, FEB SX and the hybrid derivatives 10 and 12. The initial conditions (70:15:15) were used during 10 min and modified to 42:9:49 in 5 min. Under these conditions, the retention times for IS, FEB SX, 12, FEB and 10 were: 7.8, 8.7, 15.8, 17.4 and 20.3 min, respectively. A mobile phase of acetonitrile:water (20:80) in isocratic mode, at a flow rate of 1 ml/min, with detection at 210 nm, was conducted for compound 2 (valerolactam derivative), as reported (Munguía et al., 2013). Under these chromatographic conditions, the retention times for compound 2 and IS were 6.06 and 10.84 min respectively.

Data and chromatograms were collected and analyzed using the Empower System program Waters Corporation, 2002. Quantification of drugs was done according to what we have previously described (Munguía et al., 2013) with minor modifications, by spiking known amounts in parasite material and repeating the whole process described earlier (by quintuplicate).

Recovery of compounds was estimated by comparing the peak areas from spiked parasite material samples with the areas resulting from direct injections of standards in methanol. Blank samples of drug-free parasites were incubated under the same conditions. Linearity was established from calibration curve using least-squares linear regression analysis and the correlation coefficient (r) was calculated. The data were analyzed for linearity using the GraphPad Prism 6.0 software (trail version, GraphPad Software, Inc.) minimizing sum-of-squares. Accuracy and precision were determined by processing six replicates of drug-free samples spiked with known amounts of compounds (valerolactam derivative, benzimidazole carbamates or hybrid compounds) and expressed as coefficient of variation (CV). The total amount of compound inside the parasites was expressed as nmol/100 mg of protein.

2.7. Statistical analysis

The reported data are expressed as mean ± S.D. (five replicates). An ANOVA test was conducted (once normal distribution of data was confirmed) followed by Tukey's *post hoc* test of multiple comparison of data, with 95% confidence (GraphPad Prism 6.0 trial version, La Jolla, CA, USA), a probability of $P < 0.05$ was considered statistically significant.

3. Results

The clean-up, extraction and analytical methods for *M. vogae* samples were adequate for all the compounds analyzed, with a mean recovery in the range of 63.3–117.5%, CV ≤ 12.3% (accuracy) and the linearity (r) in the range of 0.95–0.99 (see Appendix: Supplementary material). The results of determination of intraparasitic drug concentrations after *ex vivo* diffusion experiments in *M. vogae* are shown in Table 1. Results were compared for each hybrid compound and their benzimidazole anthelmintic analogous and valerolactam derivative 2. From this analysis, significant differences for hybrid compounds 8 and 10 were found, reaching higher intraparasitic concentrations compared to valerolactam derivative 2, and significantly lower concentrations compared to their parent commercial benzimidazoles (ABZ and FEB respectively) were observed.

Interestingly, the formation of sulphoxidized metabolites (ABZ SX and FEB SX) was observed during intraparasitic *ex vivo* diffusion studies of ABZ and FEB in *M. vogae*, respectively. In fact, 4.34 ± 1.11 nmol/100 mg protein of ABZ SX and 11.08 ± 3.58 nmol/100 mg protein of FEB SX were measured, corresponding to percentages of metabolite formation of 8.2% and 14.5%, respectively (not shown).

For *H. contortus* parasite material, the clean-up, extraction and analytical methods of samples were also adequate for the compounds analyzed, with a mean recovery in the range of 64.3–106.6%, CV ≤ 17.0% (accuracy) and the linearity (r) in the range of 0.96–0.99 (see Appendix: Supplementary material).

As described in Section 2 buffer phosphate (pH 7.8) was used for *H. contortus* parasite sample preparation, instead of water as in *M. vogae*, to improve the recovery of compounds. However, for ABZ and its hybrid compound 8, a very important oxidation effect was observed during sample preparation under the experimental conditions applied for *H. contortus* parasite to perform *ex vivo* diffusion experiments, which was studied before. In fact, blank samples containing drugs and phosphate buffer instead of parasites were processed and analyzed similarly as described earlier. The amount of sulphoxides non-enzymatically formed for ABZ and its hybrid compound 8 was observed and measured. These amounts were subtracted from the corresponding amounts of sulphoxides detected in parasite samples during *ex vivo* diffusion experiments in *H. contortus*. In any case, and even considering this correction, an important oxidative parasite matrix-effect (both *Hc S* and *Hc U*) was observed and determined for those two compounds (ABZ and its hybrid compound 8). The amounts of sulphoxide metabolites formed (expressed as percentage of oxidation of the parent compound) were 46% and 62% for ABZ and 8, respectively, in *Hc U*; and 37.7% and 67.3% for ABZ and 8, respectively, in *Hc S*.

Neither oxidative effect (non-enzymatically and *H. contortus* parasite-matrix) was observed for FEB or for its hybrid compound 10 during sample preparation under the same experimental conditions.

The results of total amount of intraparasitic concentration of compounds studied from *ex vivo* diffusion in *H. contortus (Hc U)* are shown in Fig. 2. No differences were observed for diffusion experiments of compounds tested using parasites derived from the different sheep farms studied. The sulphoxide metabolites produced (ABZ SX, compound 11, and FEB SX) during diffusion studies were analyzed and added to the amount of parent drug (total amount of drug). Interestingly, no formation of the sulphoxide metabolite of hybrid 10 (compound 12) was observed during the diffusion study of this compound under these experimental conditions. Valerolactam

Table 1
Intraparasitic *ex vivo* diffusion in *M. vogae TT*. Intraparasitic concentration of compounds expressed as nmol/100 mg protein found after 30 min *ex vivo* incubation of cestodes with 5 nmol/ml of each product (final concentration).

Product	Intraparasitic concentration (mean ± S.D.)
ABZ	52.41 ± 3.64[*,a]
8	35.51 ± 3.63[a,b]
FEB	75.36 ± 9.41[*,c]
10	32.41 ± 3.89[c,b]
FLU	34.26 ± 6.89[d]
9	6.14 ± 1.50[d]
2	2.90 ± 0.48[b]

Mean from five replicates; S.D.: standard deviation.
Rows with the same letter are significantly different ($p \leq 0.05$) with 95% confidence (Tukey test).
[*] Total amount of compound (including sulphoxide metabolites expressed as parent drug); statistical analysis was performed according to Section 2.7 showing comparisons between each hybrid molecule and its precursors (commercial benzimidazole and valerolactam 2).

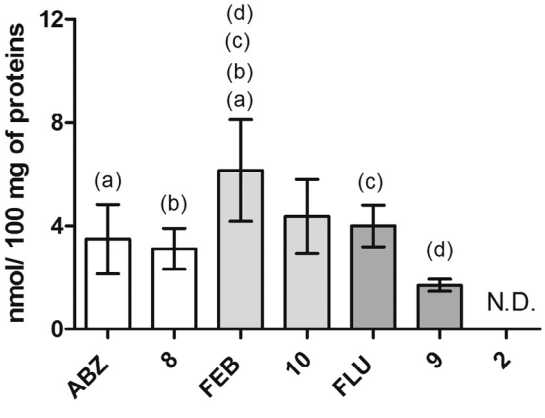

Fig. 2. Intraparasitic *ex vivo* diffusion in *H. contortus* (*Hc U*). Adult parasites were recovered from sheep abomasa of naturally infected animals from slaughterhouse (see Section 2). The bars represent the mean ± S.D. from five replicates. The intraparasitic concentration of compounds (expressed as nmol/100 mg protein) was found after 30 min *ex vivo* incubation of nematodes with 5 nmol/ml of each compound (final concentration). Bars with the same letter are significantly different ($p \leq 0.05$); N.D.: not detected.

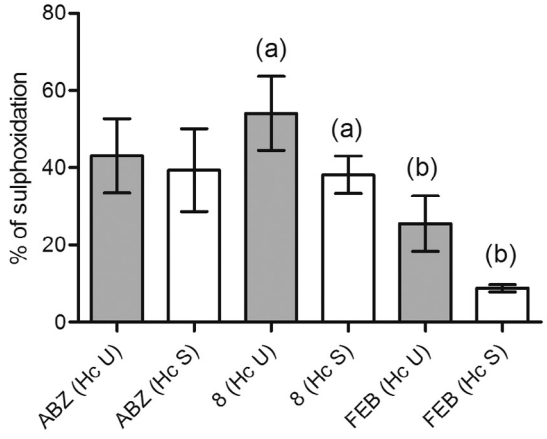

Fig. 3. Percentage of sulphoxidation during *ex vivo* intraparasitic diffusion in *H. contortus Hc U*: adult parasites recovered from sheep abomasa of naturally infected animals from slaughterhouse; *Hc S*: anthelmintic-susceptible adult parasites. Statistical analysis was performed according to Section 2.7. Bars with the same letter are significantly different ($p \leq 0.05$). See Sections 2 and 3 for further details.

compound 2 was not detected. The analysis of results revealed that there are no significant differences between intraparasitic concentrations for each hybrid compound compared to its BZ precursor, while FEB showed the highest intraparasitic concentration (6.15 ± 1.97 nmol/100 mg protein) with significant differences among the series of compounds studied (except compound 10) (Fig. 2).

Interestingly, significant differences between *Hc S* and *Hc U* adult parasites were observed when the percentage of oxidation of compounds FEB and hybrid 8 were compared (Fig. 3). In fact, the percentage of oxidation of FEB and the hybrid compound 8 were higher in *Hc U* (25.5%, 54.1% respectively) than in *Hc S* (8.8%, 38.2%), while for ABZ there were no significant differences between *Hc S* and *Hc U* adult worms.

4. Discussion

The anthelmintic activity of compounds basically depends on two aspects: their affinity for a specific receptor, and the transport properties that allow the delivery of effective concentrations of the compound in sufficient time at the target site into the parasite (Thompson et al., 1993). However, even when physiology-based *in vitro* assays mimic the physiological situation within the host, parasites are cultured in the presence of compounds for longer in comparison with the *in vivo* situation (*e.g.* one oral dose). In summary, both the parasite and host pharmacokinetic aspects of a drug must be considered jointly to better understand the response to an anthelmintic treatment. Thus, a bioactive *in vitro* compound with a slow rate of intraparasitic diffusion could simulate good bioavailability into parasite. We have demonstrated this for a new valerolactam derivatives series (compound 2) using rat nematode *N. brasiliensis* L4. Indeed, those compounds showed extraordinary *in vitro* anthelmintic activity, but did not penetrate parasite barrier and showed lack of efficacy in the *in vivo* bioassay (Munguía et al., 2013).The design and synthesis of the novel valerolactam-benzimidazole hybrids (bioactive in the *in vitro* model), and the improved physicochemical properties compared with the valerolactam domain allowed the penetration of *N. brasiliensis* barriers, concurrently with an *in vivo* activity in the *N. brasiliensis* rat model (Domínguez et al., 2000; Munguía et al., 2013).

H. contortus, a target nematode in sheep, and the cestode *M. vogae* were selected as parasite models in this work to further study those novel hybrid molecules. BZ anthelmintics reach helminth parasites principally by passive diffusion through the external surface, either cuticle in nematodes or tegument in cestodes, even considering the differences in morphological and functional properties of the parasite's external surfaces (Alvarez et al., 2007; Mottier et al., 2003, 2006). The cestode tegument is a surface which shows morphological and biochemical similarities with mammalian gut mucosa (Lumsden, 1975; Thompson and Geary, 1995). The nematode's cuticle is a potential site for drug uptake, in which the aqueous and lipidic pores of the collagen matrix control the passage of molecules depending on their physicochemical properties (Ho et al., 1990, 1992).

In this sense, the differences observed between diffusion studies of compounds showed significantly higher intraparasitic concentrations in *M. vogae* compared with *H. contortus* (Table 1 and Fig. 2). In fact, an average of nine times higher intraparasitic concentration of compounds was measured in *M. vogae* compared to *H. contortus* (a range with a maximum of 15 times higher for ABZ and minimum of 3.6 times higher for hybrid compound 9, in *M. vogae*).

These results agree with previous reports (Mottier et al., 2006) in which FEB diffusion studies revealed significantly lower intraparasitic concentrations measured inside nematode parasite (*Ascaris suum*) than those obtained in cestodes (*Moniezia benedeni*), even at a longer incubation time (90 min) than those used in the experimental conditions of this work.

In addition, a good correlation (r^2: 0.89) between lipophilicity (expressed as capacity factor φ_0 determined previously by our group, Munguía et al., 2013) and intraparasitic concentration of hybrid derivatives was observed in *H. contortus*. In fact, hybrid compound 10, the most lipophilic (φ_0: 67.1) of the hybrid series, showed the highest intraparasitic drug concentration measured (4.36 ± 1.44 nmol/100 mg protein). However, we did not find a good correlation using this parameter (lipophilicity) when benzimidazole carbamates were also included with their hybrid compounds in the series. It is noted that lipophilicity as a sole parameter cannot accurately predict how rapidly the molecules are absorbed. In fact, penetration of the parasite barriers depends also on the molecular size and electrical charge of the permeant (Thompson and Geary, 1995). Both cuticle of nematodes and tegument of cestodes (Ho et al., 1992; Knowles and Oaks, 1979) show a negative charge on the surface of the parasite, therefore, greater dipole moment of molecules could result in repulsion effects, and concomitantly poor intraparasitic diffusion in both nematode and cestode parasites. Accordingly, other geometric parameters like molecular volume and dipole moment should also be considered. Studies to find the best correlation with diffusion capacity are currently in progress.

On the other hand, some interesting differences regarding oxidation pattern of compounds studied in the helminth targets selected were observed.

Sulphoxidation was confirmed for ABZ and FEB in both *H. contortus* and *M. vogae* diffusion studies. No oxidation was observed for hybrid compounds in *M. vogae* studies, and only hybrid compound 8 (among hybrid compounds) was oxidized in *H. contortus*. This effect was more relevant in *H. contortus* with percentages of oxidation 5.3 and 1.8 times higher, for ABZ and FEB, respectively, compared with those in *M. vogae*.

An interesting difference was observed in this pattern between *H. contortus* anthelmintic-susceptible adult parasites (*Hc S*) and unknown adult parasites recovered from sheep farms (*Hc U*) which were used under the assumption that they are resistant to BZ compounds. In fact, the percentages of oxidation of FEB and hybrid compound 8 were higher in *Hc U* (25.5%, 54.1%, respectively) than in *Hc S* (8.8%, 38.2%). Oxidation of hybrid compound 10 was not observed either in *Hc S* or in *Hc U* diffusion studies.

As the oxidative/reductive pathways in detoxification of xenobiotics (phase I) by helminth parasites are unclear (Cvilink et al., 2009; Kotze et al., 2006), their participation in resistance development is even more uncertain. In this sense, some previous studies were addressed, particularly for BZ, to specify the enzymes involved, by identifying the metabolites formed by parasites. Under this approach, the ability

of different helminth parasites to metabolize BZ was studied (Cvilink et al., 2008; Solana et al., 2001; Vokřál et al., 2013).

In this work we have demonstrated and measured the amount of sulphoxides produced by chemical process (blank sample without parasites) and by parasites (matrix-effect) during the *H. contortus* sample preparation for ABZ and hybrid compound 8. This observation of non-enzymatically ABZ sulphoxide formed during sample parasite preparation was also communicated before, to justify the corrections made to the total amount of this metabolite (Vokřál et al., 2013).

In addition, this effect was found selectively for ABZ and its hybrid 8. In this sense, the study of the influence of different conditions (pH, solvents, presence or absence of oxygen, etc.) on the oxidation reaction (chemical and enzymatic) could contribute to the search for the enzymes involved (studies under way).

5. Conclusions

The novel hybrid valerolactam-benzimidazole molecules have shown improved intraparasitic concentrations with respect to valerolactam moiety and comparable concentrations with respect to their BZ in parasite models studied, *H. contortus* adult nematode and *M. vogae* cestoda. Even considering the differences between external parasite surfaces, which could explain greater amount of drugs measured inside the cestode parasite with respect to nematode parasite, some similarities regarding the order of entry of compounds inside the parasites were found. In fact, valerolactam compound 2 was not found or was practically insignificant, while FEB and its hybrid compound 10 (particularly in *H. contortus*) showed the highest intraparasitic concentrations measured under the experimental conditions described. No differences in the total amount of drugs inside the *H. contortus* adult parasites between anthelmintic-susceptible (*Hc S*) and those derived from sheep farms (*Hc U*) were found. However, significant differences in the percentage of oxidation of FEB and hybrid compound 8 were found between *Hc S* and *Hc U*. Even more interesting was the finding that sulphoxidation was not observed for hybrid compound 10, neither for *Hc S* nor for *Hc U* adult parasites, indicating that increased drug metabolism (phase I) produced by the action of xenobiotic metabolizing enzymes could not be used by this parasite as protection against this novel drug. Further studies must be conducted to confirm these observations.

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.exppara.2015.03.013.

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