Alternative energy production pathways in *Taenia crassiceps* cysticerci *in vitro* exposed to a benzimidazole derivative (RCB20)

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SUMMARY

Biochemical studies of benzimidazole derivatives are important to determine their mode of action and activity against parasites. The lack of antihelminthic alternatives to treat parasitic infections and albendazole resistance cases make the search for new antiparasitary drugs of utmost importance. The 6-chloro-5-(1-naphthyloxy)-2-(trifluoromethyl)-1H-benzimidazole (RCB20) is a benzimidazole derivative with promising effect. This study evaluated the effect of different concentrations of RCB20 in the alternative energetic pathway of *in vitro Taenia crassiceps* cysticerci. The parasites were *in vitro* exposed to 6·5 and 13 μM of RCB20 and albendazole sulfoxide (ABZSO). The quantification of acetate, acetoacetate, β-hydroxybutyrate, fumarate and propionate was performed by high-performance liquid chromatography. The quantification of urea, creatinine and total proteins was performed by spectrophotometry. The increase in β-hydroxybutyrate reflects the enhancement of the fatty acid oxidation in the treated groups. Volatile fatty acids secretion, acetate and propionate, was increased in the treated groups. The secretion mechanisms of the treated parasites were impaired due to organic acids increased concentrations in the cysticerci. It is possible to conclude that the metabolic effect on alternative energetic pathways is slightly increased in the parasites treated with RCB20 than the ones treated with ABZSO.

Key words: *Taenia crassiceps*, ketonic bodies, fatty acids oxidation, protein catabolism, benzimidazole derivative.

INTRODUCTION

The most used drugs on the treatment of parasitic infections are benzimidazoles such as albendazole, fenbendazole, oxfendazole, mebendazole and triclabendazole. These drugs affect both intestinal and tissue parasites due to their mode of action which is the blockage of microtubule and tubulin formation within the parasite's cell (Barot *et al*. 2013). This blockage results in several biochemical effects within the parasite which contribute to its death, such as glucose uptake impairment, induction of alternative energetic pathways and consequent death by starvation (Vinaud *et al*. 2007; Fraga *et al*. 2012*a*; Márquez-Navarro *et al*. 2013). Few benzimidazole derivatives have been studied with regard to their biochemical effect, for instance, albendazole, cambendazole, oxibendazole and thiabendazole dissipate the transmembrane proton gradient resulting in diminished ATP levels (McCracken and Stillwell, 1991). The biochemical studies of benzimidazole derivatives may help to understand their mode of action and to determine their specificity within the metabolic pathways that are essential for the parasite's survival (McCracken and Stillwell, 1991; Feldmeier, 2010; Geary, 2012).

Worldwide Taeniidae parasites still remain important public health issues which make them the target of several studies such as biochemical, physiological, susceptibility to drugs and others (Hoberg *et al*. 2000). Due to their strikingly high incidence both in humans and in animals these parasites are important indicators of health and sanitary conditions of the population (Aragão *et al*. 2010; Coral-Almeida *et al*. 2015; Scala *et al*. 2015).

The most common drugs used against helminthes are albendazole and praziquantel. Their mode of action is related to tegument damage, calcium

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channels impairment and inhibit of β-tubulin polimerization. These effects lead to metabolic effects such as glucose uptake impairment and the induction of anaerobic metabolism and alternative pathways for energy production (Venkatesan, 1998; Vinaud *et al*. 2007, 2008, 2009).

Albendazole has been widely used as anti-helminthic treatment against intestinal and tissue parasites (Feldmeier, 2010; Del Brutto, 2014). However, due to its use for longer than 20 years, albendazole resistance reports have been made (Márquez-Navarro *et al*. 2012; Lopes *et al*. 2014). Therefore studies aiming to improve the efficiency of the albendazole molecule and its mode of action have been conducted (Márquez-Navarro *et al*. 2013; Matadamas-Martínez *et al*. 2013; Garćia *et al*. 2014). One albendazole derivative has evidenced promising activity – the 6-chloro-5-(1-naphthyloxy)-2-(trifluoromethyl)-1H-benzimidazole (RCB20). This derivative presented a better *in vitro* activity on *Taenia crassiceps* cysticerci than sulfoxide albendazole (ABZSO) regarding morphological parameters (Márquez-Navarro *et al*. 2013).

The fatty acids oxidation, ketone body production and urea cycle are metabolic pathways described in *T. crassiceps* and other cestodes used for energy production and essential for the parasite's survival. The parasite removes from the environment or from reserve vacuoles the substrates for these pathways with considerable ATP production. In situations of environmental stress such as the presence of antihelminthic drugs that impair the glucose uptake the parasite enhances these metabolic pathways enabling its survival (Vinaud *et al*. 2009; Fraga *et al*. 2012*b*). Therefore the studies of the metabolic range of the parasites when exposed to drugs may indicate the pathways used to escape metabolic distress and a possible resistance development (Barot *et al*. 2013).

As albendazole is capable of *in vitro* and *in vivo* enhancing the use of alternative pathways for energy production by *T. crassiceps* cysticerci (Vinaud *et al*. 2009; Fraga *et al*. 2012*a*), this study aimed the evaluation of the fatty acid oxidation and urea cycle in *T. crassiceps* cysticerci *in vitro* exposed to RCB20, considering that it is a molecule similar to albendazole with different chemical properties.

MATERIALS AND METHODS

Maintenance of T. crassiceps cysticerci

The maintenance of *T. crassiceps* cysticerci was performed according to the description by Fraga *et al*. (2012*a*). The ethical principles of animal experimentation stipulated by the Brazilian Society of Laboratory Animals Science (SBCAL) were obeyed. This study was approved by the Ethics in Research Committee from the Federal University of Goias (CoEp/UFG) (protocol number 011/11).

Cysticerci culture and drugs exposure

A mouse with 30 days of intraperitoneal infection was euthanized within a laminar flow chamber and the cysticerci were removed and washed with physiological solution as to remove cells and any other contaminants (Márquez-Navarro *et al*. 2013). Afterwards the cysticerci were macroscopically classified into its evolutive stages: initial (no buds, vesicular membrane and fluid translucent), larval (with buds, vesicular membrane and fluid translucent) and final (no buds, vesicular membrane and fluid opaque) (Vinaud *et al*. 2007).

The cysticerci culture was performed as described by Vinaud *et al*. (2008) and Márquez-Navarro *et al*. (2013). Briefly, 30 cysticerci from each evolutive stage were added into 5 mL of supplemented RPMI culture medium. The treatments were as follows: albendazole sulfoxide (ABZSO) (Sigma-Aldrich®) (6·5 and 13 μM) and RCB20 (6·5 and 13 μM). The control groups of each evolutive stage did not receive the drugs. There were other control groups of each evolutive stage that received dimethyl sulfoxide (DMSO) at the same concentration used to dilute the drugs.

After 24 h of culture the cysticerci were separated from their culture medium and frozen into liquid nitrogen as to stop the metabolic reactions (Vinaud *et al*. 2008, 2009).

Biochemical analysis

The organic acids secreted/excreted into the culture medium were extracted through an ionic exchange solid phase extraction column (Bond Elut® Agilent®), as described by Vinaud *et al*. (2007).

After the liquid nitrogen metabolic stasis, the cysticerci were defrost and homogenized in 500 μL of tris–HCl 0·1 M buffer supplemented with a protease inhibitor (SIGMAFAST, protease inhibitor cocktail tablets, EDTA free, Sigma), pH 7·6 (Rendón *et al*. 2004, 2008). The extract obtained was centrifuged at 15 652 ***g*** (10 000 rpm) per 10 min at 4 °C and then the organic acids were extracted through an ionic exchange solid phase extraction column (Bond Elut® Agilent®) (Vinaud *et al*. 2007).

The resulting samples were frozen at −20 °C for posterior analysis in high-performance liquid chromatography.

For the chromatographic analysis an exclusion BIORAD-Aminex HPX-87H column was used. The eluent was sulphuric acid 5 mM, 0·6 mL min^{-1}, with spectrophotometric reading of absorbance at 210 nm. The results were analysed through the Star Chromatography Workstation software (Agilent®), previously calibrated for the following organic acids identification: acetate, acetoacetate, β-hydroxybutyrate, propionate and fumarate (Vinaud *et al*. 2007). The analysis of the proteins catabolism was performed through the quantification of urea,

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creatinin and total proteins in the culture medium which was performed through an Architec C8000 Plus device, using a commercial kit protocol and enzymatic method (Fraga *et al*. 2012*b*).

Statistical analysis

All experiments were repeated five times independently. The statistical analysis was performed through the Sigma Stat 2·3 software. The descriptive analysis was performed as to determine the normal distribution and homogenous variation as well as mean and standard deviation. As the values presented normal distribution, the analysis-of-variation test (ANOVA) was performed. The differences were considered significant when $P < 0{\cdot}05$.

RESULTS

This study showed the metabolic effect of two benzimidazole derivatives (RCB20 and ABZSO) in a widely used experimental model, *T. crassiceps* cysticerci. It was possible to evaluate both the quantification of organic acids within the cysticerci and in the culture medium (secreted/excreted by the parasite) in three different evolutive stages of the parasite, initial, larval and final ones. It was possible to detect the fatty acid oxidation and the protein catabolism in all cysticerci stages through the quantification of acetate, acetoacetate, β-hydroxybutyrate, propionate, fumarate, urea, creatinin and total proteins (Tables 1 and 2). Both RCB20 and ABZSO were diluted in DMSO, which did not interfere in the parasite's metabolic pathways.

Fatty acid oxidation

The acetate concentrations were detected mostly in the culture medium and not within the cysticerci. The detection of this organic acid in the cysticerci occurred only in initial stages ones with RCB20 6·5 and ABZSO 13 μM.

It was not possible to detect acetoacetate in the control group, i.e., which did not receive any treatment, neither in the cysticerci nor in the culture medium. However in the treated groups, this organic acid was detected in the cysticerci from all stages (treated with ABZSO and RCB20 at 6·5 μM), initial stage (treated with RCB20 13 μM) and final stage (treated with ABZSO 13 μM).

The β-hydroxybutyrate detection occurred mainly in the culture medium analysis of the initial and larval stages cysticerci from the control group and the ones treated with both drugs at 6·5 μM. In the final stages cysticerci, it was not possible to detect this organic acid neither in the culture medium nor in the cysticerci with the exception of the group treated with RCB20 13 μM which presented a higher concentration in the culture medium than in the cysticerci. In the other treated groups, when this organic acid was detected its concentration was higher in the cysticerci than in the culture medium.

The propionate detection occurred mainly in the culture medium with the exception of initial stages cysticerci both from the control group and treated ones. The propionate concentrations detected in the cysticerci analysis of the initial stage ones treated with both concentrations of ABZSO were significantly higher than in the culture medium ($P < 0{\cdot}05$). In the larval and final stage cysticerci treated with RCB20 13 μM, propionate was not detected neither in the cysticerci nor in the culture medium.

Proteins catabolism

It was only possible to analyse the creatinin, urea and total proteins concentrations on the culture medium. All cysticerci analysed presented a functional urea cycle which was detected through the quantification of fumarate, creatinin, urea and total proteins. There was no significant difference in the creatinin and total proteins quantification. On the other hand, the urea concentrations detected in the culture medium were increased in final stage cysticerci treated with ABZSO 6·5 μM when compared with the same evolutive stage in the control group. The RCB20 treatment did not influence the proteins catabolism. The fumarate concentrations were higher in all the analysis of the cysticerci when compared with the culture medium ($P < 0{\cdot}05$) (Table 2).

DISCUSSION

This study evaluated the *in vitro* metabolic effect of two benzimidazole derivatives on the alternative energetic pathways of *T. crassiceps* cysticerci, an experimental model widely used for metabolic analysis of Taeniidae (Vinaud *et al*. 2007, 2008, 2009; Fraga *et al*. 2012*a*, *b*; Márquez-Navarro *et al*. 2013). The need of benzimidazole derivatives have been explored in the literature with promising results (Barot *et al*. 2013) due to the extensive use of albendazole and the appearance of resistance cases, especially within veterinary use (Geary, 2012; Márquez-Navarro *et al*. 2012).

The acetoacetate detection occurred mainly in the cysticerci of the treated groups because it is the β-hydroxybutyrate precursor (Tielens *et al*. 2010) and also because in those groups probably a greater fatty acid oxidation occurred. Also in some groups (control, RCB20 13 μM) it was not possible to detect this organic acid, but the β-hydroxybutyrate detection occurred. This happened due to the consumption of acetoacetate to β-hydroxybutyrate production. On the other hand, the treatment with 6·5 μM concentrations of both RCB20 and ABZSO

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Table 1. Mean concentrations (μM) of organic acids of the fatty acid oxidation and volatile fatty acids detected in the medium culture (secreted/excreted) and in the cysticerci of *Taenia crassiceps in vitro* exposed to ABZSO and RCB20. Results expressed in mean ± standard deviation

	Acetoacetate		β-hydroxybutyrate		Acetate		Propionate	
Cysticerci evolutive stage	CM	Cy	CM	Cy	CM	Cy	CM	Cy
Control								
Initial	ND	ND	114·60 ± 47·11	ND	ND	ND	63·01 ± 35·77	54·54 ± 5·37
Larval	ND	ND	450·42 ± 489·10	2307·83 ± 1972·94*	ND	ND	21·07 ± 1·94	ND
Final	ND	ND	ND	ND	ND	ND	25·07 ± 9·53	ND
Control – DMSO								
Initial	ND	ND	92·49 ± 57·00	ND	ND	ND	137·76 ± 3·33	ND
Larval	ND	ND	513·07 ± 712·11	514·18 ± 96·61	ND	ND	41·05 ± 12·28	ND
Final	ND	ND	ND	ND	ND	ND	17·53 ± 2·34	ND
RCB20 6·5 μM								
Initial	ND	52·04 ± 34·51	116·51 ± 66·69	284·77 ± 210·46*	72·04 ± 37·01	75·89 ± 1·65	120·18 ± 65·17	59·26 ± 14·83
Larval	ND	88·49 ± 17·68	508·34 ± 506·13	3637·76 ± 692·89*	285·23 ± 131·11	ND	59·09 ± 30·34	ND
Final	ND	319·53 ± 265·12	ND	ND	512·32 ± 118·77	ND	39·63 ± 28·96	ND
RCB20 13 μM								
Initial	ND	30·91 ± 10·9	122·21 ± 84·44	ND	65·75 ± 21·35	ND	74·81 ± 49·04	ND
Larval	ND	ND	79·14 ± 60·39	4160·10 ± 612·36*	237·08 ± 153·44	ND	ND	ND
Final	ND	ND	640·90 ± 328·33	270·05 ± 9·73	440·33 ± 176·88	ND	ND	ND
ABZSO 6·5 μM								
Initial	ND	48·47 ± 36·92	ND	ND	100·79 ± 108·04	ND	41·13 ± 11·27	104·99 ± 39·67*
Larval	ND	131·82 ± 91·04	164·20 ± 102·47	298·41 ± 191·20*	258·00 ± 130·87	ND	32·21 ± 16·47	ND
Final	ND	653·03 ± 175·44	ND	ND	649·07 ± 334·33	ND	ND	ND
ABZSO 13 μM								
Initial	ND	ND	118·48 ± 128·09	ND	28·25 ± 8·51	ND	30·31 ± 14·14	77·76 ± 28·01*
Larval	ND	ND	90·88 ± 50·89	167·54 ± 150·00*	197·94 ± 65·74	ND	221·77 ± 312·18	ND
Final	ND	240·96 ± 288·44	ND	ND	473·47 ± 59·15	ND	53·91 ± 4·73	ND

CM, culture medium; Cy, cysticerci; ND, non-detected; RCB20, 6-chloro-5-(1-naphtyloxy)-2-(trifluorometil)-1*H*-benzimidazol; ABZSO, albendazole sulfoxide.
* Statistical difference when compared to the concentrations detected in the culture medium (*t* test, $P < 0{\cdot}05$).

Table 2. Mean concentrations of fumarate (μM), urea (mg dL^{-1}), creatinin (mg dL^{-1}) and total proteins (g dL^{-1}) in the culture medium of *Taenia crassiceps* cysticerci *in vitro* exposed to ABZSO and RCB20. Results expressed in mean ± standard deviation

Cysticerci evolutive stage	Fumarate (μM)	Urea (mg dL^{-1})	Creatinin (mg dL^{-1})	Total proteins (g dL^{-1})
	Cy / CM	CM	CM	CM
Control				
Initial	23·38 ± 4·26[a]	5·43 ± 1·73	7·66 ± 2·33	0·23 ± 0·05	0·51 ± 0·14
Larval	187·49 ± 130·69[a]	25·19 ± 19·03	10·50 ± 2·25	0·25 ± 0·05	0·60 ± 0·08
Final	785·71 ± 456·87[a]	30·62 ± 32·74	11·00 ± 3·5	0·30 ± 0·15	0·56 ± 0·10
Control – DMSO				
Initial	15·01 ± 7·88[a]	4·86 ± 3·23	7·83 ± 2·64	0·23 ± 0·08	0·55 ± 0·14
Larval	311·31 ± 264·66[a]	24·88 ± 16·54	9·67 ± 1·37	0·23 ± 0·05	0·57 ± 0·12
Final	601·01 ± 173·16[a]	42·58 ± 34·80	9·83 ± 1·83	0·23 ± 0·05	0·58 ± 0·10
RCB20 6·5 μM				
Initial	36·56 ± 25·49[a]	6·06 ± 3·48	7·83 ± 2·22	0·23 ± 0·05	0·56 ± 0·10
Larval	190·58 ± 102·38[a]	24·44 ± 21·00	10·83 ± 2·71	0·25 ± 0·05	0·65 ± 0·10
Final	650·16 ± 203·90[a]	36·28 ± 40·06	10·66 ± 2·50	0·26 ± 0·05	0·65 ± 0·05
RCB20 13 μM				
Initial	37·04 ± 21·93[a]	5·79 ± 4·11	8·00 ± 2·19	0·21 ± 0·09	0·56 ± 0·10
Larval	455·80 ± 388·94[a]	23·91 ± 15·75	10·50 ± 2·07	0·26 ± 0·05	0·63 ± 0·10
Final	585·23 ± 231·89[a]	31·40 ± 29·62	10·00 ± 1·09	0·23 ± 0·05	0·60 ± 0·06
ABZSO 6·5 μM				
Initial	25·36 ± 13·62[a]	4·52 ± 3·32	8·50 ± 2·81	0·26 ± 0·05	0·60 ± 0·12
Larval	404·25 ± 231·61[a]	25·20 ± 13·60	11·50 ± 2·58	0·25 ± 0·05	0·61 ± 0·07
Final	846·25 ± 484·19[a]	41·55 ± 43·09	**12·66 ± 2·65***	0·25 ± 0·05	0·65 ± 0·08
ABZSO 13 μM				
Initial	19·98 ± 2·92[a]	5·58 ± 2·75	7·83 ± 2·48	0·25 ± 0·08	0·60 ± 0·14
Larval	287·97 ± 144·95[a]	38·12 ± 21·34	10·50 ± 3·20	0·20 ± 0·06	0·58 ± 0·11
Final	719·30 ± 224·15[a]	43·33 ± 38·68	10·83 ± 1·83	0·25 ± 0·05	0·63 ± 0·05

RCB20, 6-chloro-5-(1-naphtyloxy)-2-(trifluorometil)-1*H*-benzimidazol; ABZSO, albendazole sulphoxide; Cy, cysticerci; CM, culture medium.
[a] Statistical difference when compared with the concentrations detected in the culture medium (*t* test).
Bold and *: statistical difference when compared with the control group (ANOVA).

induced a greater production of this organic acid in which the β-hydroxybutyrate production was also increased both in the culture medium and in the cysticerci analyses. Probably this happened due to a greater oxidation of fatty acids in the cysticerci exposed to those concentrations. This metabolic response in *T. crassiceps* cysticerci *in vivo* and *in vitro* exposed to albendazole was also described by Vinaud *et al*. (2009) and Fraga *et al*. (2012*b*). Also the higher detection of β-hydroxybutyrate in the cysticerci of the treated groups when compared with the control ones may happen due to the mode of action of RCB20 and albendazole which alters the tubulin formation impairing the secretion mechanisms of the cells (Márquez-Navarro *et al*. 2013). Therefore the whole secretion mechanism of the parasite is affected. On the other hand, acetate and propionate which are volatile fatty acids do not need tubulins to be carried out of the cytoplasm (Ahring *et al*. 1995) and that is why they were mainly detected in the culture medium analysis (secreted/excreted by the parasite).

Also as propionate is the end product of the succinate fermentation via succinyl-, methylmalonyl- and propionyl-coenzime A (Reichardt *et al*. 2014), this pathway indicates energy production by the parasite which is increased in initial stage cysticerci treated with ABZSO 6·5 and 13 μM.

The fumarate detection was higher in the cysticerci than in the culture medium as it is an organic acid which participates both in the urea cycle and in the tricarboxilic acid cycle. Therefore its secretion/excretion occurs only when it is in excess. As the parasites were in the culture medium with great amounts of glucose, amino acids and fatty acids all the energetic pathways were available to them (Tielens *et al*. 2010). Also the drugs, in the concentrations tested in this study, did not interfere in the urea cycle.

The urea cycle was also detected in *T. crassiceps* cysticerci as an energy production pathway from the proteins catabolism. Such cycle has been described previously in this parasite by Vinaud *et al*. (2009) in *in vitro* studies and by Fraga *et al*. (2012*a*) in *in vivo* studies. It is important to highlight that the parasite is capable of performing simultaneously several energetic rentable pathways such as glycolysis, the pentose phosphate pathway, the tricarboxylic acid cycle, the urea cycle, the fatty acids oxidation, among others (Barret, 2009; Tielens

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et al. 2010). In addition when it is metabolic challenged, both *in vivo* and *in vitro*, some of these pathways are enhanced or decreased (Vinaud *et al*. 2007, 2008, 2009; Fraga *et al*. 2012*a*, *b*).

The concentrations of ABZSO and RCB20 used in this study were the ones described by Márquez-Navarro *et al*. (2013) in which structural effects were observed in *in vitro T. crassiceps* cysticerci. In spite of the morphological alterations and the different expression of tubulin isoforms these concentrations do not cause great biochemical alterations in the metabolic pathways, only in the secretion/excretion mechanism. Further studies with higher concentrations in *in vivo* analysis should be performed as to confirm RCB20 as an alternative benzimidazolic drug.

It is possible to infer that the structural alteration in the RCB20 molecule when compared with the ABZSO one does not interfere in its metabolic effect acting as a promising alternative to albendazole as the biochemical effects occur in the same targets. Therefore we conclude that the metabolic effects on alternative energetic pathways are slightly increased in the parasites treated with RCB20 than the ones treated with ABZSO.

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