Asian Pacific Journal of Tropical Disease

journal homepage: http://www.apjtcm.com

Original article https://doi.org/10.12980/apjtd.7.2017D7-256

Benznidazole induces *in vitro* anaerobic metabolism in *Trypanosoma cruzi* epimastigotes

Marina Clare Vinaud*, Kamilla Soares Nogueira, Carolina Miguel Fraga, Tatiante Luiza da Costa, Ana Maria de Castro

Tropical Pathology and Public Health Institute, Federal University of Goias, Goiás, Brazil

ARTICLE INFO

Article history:
Received 8 Nov 2017
Received in revised form 16 Nov 2017
Accepted 24 Nov 2017
Available online 29 Nov 2017

Keywords:
Antiparasitic drug
Chagas disease
Anaerobic metabolism
Benznidazol

ABSTRACT

Objective: To determine the biochemical alterations of the energetic metabolism of *Trypanosoma cruzi* epimastigotes *in vitro* exposed to different concentrations of benzinidazole.
Methods: Biochemical analyses were performed at 3, 6 (log phase), 9 and 12 (stationary phase) days of culture. Parasites were exposed to five concentrations of benzinidazole. Glycolysis, tricarboxilic acid cycle and fatty acids oxidation pathways were quantified through chromatography. Glucose, urea and creatinine were quantified through spectrophotometric analysis.
Results: Anaerobic fermentation and fatty acids oxidation were increased in the stationary phase of the culture. Benzinidazole at high concentrations induced anaerobic metabolism in the log phase of the culture while the parasites exposed to the lower concentrations preferred the citric acid cycle as energy production pathway. Benzinidazole did not influence on the proteins catabolism.
Conclusions: It is possible to conclude that there are metabolic differences between evolutive forms of *Trypanosoma cruzi* and the main drug used for its treatment induces the anaerobic metabolism in the parasite, possibly impairing the mitochondrial pathways.

1. Introduction

American trypanosomiasis also known as Chagas disease is a tropical neglected disease endemic in 21 Latin America countries. This disease afflicts more than 6 million people around the world[1]. In spite of the great effort in the identification of new drug targets and in the development of new active molecules against this parasite there are only two drugs available for its treatment – benzinidazole and nifurtimox[2].

Benzinidazole (N-benzyl-2-nitro-1-imidazole acetamide) is the main antiparasitic drug used against *Trypanosoma cruzi* (*T. cruzi*) as it shows less toxicity to adult patients than nifurtimox[2]. Similarly to other nitroimidazolic drugs, benzinidazole has been chosen as an antiparasitic drug in spite of its undetermined mode of action[3]. Both *T. cruzi* epimastigotes and trypomastigotes are capable to biotransform this drug into its metabolites that covalently bind to DNA, proteins and lipids[4]. The catabolism of benzinidazole within the parasite is linked to activities of reductases such as aldo-keto reductase which is an enzyme involved in detoxification of the drug within the cytoplasm[5]. The determination of the metabolic effect of benzinidazole on *T. cruzi* may help to identify drug targets[6].

The glucose metabolism in trypanosomatids including *T. cruzi* has been described in its different evolutive forms such as trypomastigote, epimastigote and amastigote[7]. There are glucose transporters expressed in all its evolutive stages and the parasite may use both glucose and amino-acids such as L-proline as energy sources. After the glucose incorporation an aerobic fermentation is undertaken within the parasite and the main products of these metabolic pathways in epimastigotes are succinate and alanine[7,8]. Other products of *Trypanosoma* carbohydrate catabolism are acetate, lactate and CO_2. Acetate may also be originated from amino acids and fatty acids catabolism[9].

The metacyclogenesis is the epimastigote transformation into metacyclic trypomastigotes which are capable of infecting the vertebrate host. It has been described that *T. cruzi* spontaneously performs metacyclogenesis *in vitro* at the stationary phase, around 9–11 days of culture[10]. Although several aspects of

*Corresponding author: Marina Clare Vinaud, Tropical Pathology and Public Health Institute, Federal University of Goias, Goiás, Brazil. CEP: 74605-050.
Tel: +55 62 32096113
Fax: +55 62 32096363
E-mail: marinavinaud@gmail.com
Foundation Project: Supported by the National Counsel of Technological and Scientific Development (CNPq) (Grant No. 302159/2016-9 and 471009/2013-0).
The journal implements double-blind peer review practiced by specially invited international editorial board members.

the metacyclogenesis have been described such as morphology, infectivity and gene expression[10-12], the differences in the energetic metabolism throughout the culture growth have not yet been explored. Shah-Simpson *et al*.[13] described the glucose metabolism in epimastigotes, metacyclic trypomastigotes and amastigotes determining the differences in excretion of pyruvate, acetate and alanine. The alternative energy production pathways such as proteins catabolism and fatty acids oxidation have not been described during this process.

In order to evaluate the biochemical effect of the most used antiparasitic drug against *T. cruzi*, the aim of this study was to determine the biochemical alterations of the energetic metabolism of *T. cruzi* epimastigotes *in vitro* exposed to different concentrations of benzinidazole. Also this study evaluated the metabolic differences within different culture days of epimastigotes of *T. cruzi* during the metacyclogenesis.

2. Materials and methods

2.1. T. cruzi culture

Epimastigotes of *T. cruzi*, Y strain, were cultured in liver infusion tryptose (LIT) medium supplemented with 10% inactivated fetal bovine serum at 26 °C. Cultures were initiated by inoculating exponentially growing epimastigotes to a final concentration of 3.5×10^4 parasites/mL. The parasites were followed up through counting in a Neubauer chamber. After 12 days of culture a concentration of 13×10^6 parasites/mL was obtained.

Biochemical analyses were performed at 3, 6, 9 and 12 days of culture, corresponding to the log (3 and 6 days) and stationary (9 and 12 days) phases of the parasite's growth in culture. At each experimental day, a culture tube was centrifuged at 3000 r/min for 10 min at 4 °C, and the supernatant of the culture was frozen in liquid nitrogen for posterior analysis.

2.2. Benzinidazole exposure

At Day 0, a total of 3.5×10^4 parasites/mL were inoculated in LIT culture medium and exposed to the following concentrations of benzinidazole: 100 μmol/L, 50 μmol/L, 25 μmol/L, 12.5 μmol/L and 6.125 μmol/L. The drug was diluted in DMSO (4%). After 24 h the 100 μmol/L group was centrifuged and frozen in liquid nitrogen as described previously because the parasites were not seen in the Newbauer chamber follow up. The other experimental groups were frozen after 3 days (72 h) of exposure.

2.3. Biochemical analysis

The organic acids were extracted from the culture medium as described previously[14]. The organic acids were identified through high performance liquid chromatography (HPLC) according to the previously determined retention time and calibration. The organic acids analyzed were the ones indicating glycolysis (pyruvate, lactate), tricarboxilic acid cycle (citrate, alpha-ketoglutarate, succinate, fumarate, malate and oxaloacetate) and fatty acids oxidation (acetate, acetoacetate, beta-hydroxybutyrate and propionate). Glucose, urea and creatinine were quantified through spectrophotometric analysis in an Architect device 8000 according to the protocol of the commercial kits from Abbott®.

2.4. Statistical analysis

All experiments were repeated five times independently. The statistical analysis was performed using the Sigma Stat 3.5 software. Descriptive statistics were applied to determine the mean and standard deviation and to evaluate the differences between the groups analyzed. The variables were tested for normal distribution and homogeneous variance. As they presented normal distribution, variance analysis (ANOVA) was used followed by the Bonferroni *post-hoc* test. The comparison of two groups was performed through the student's *t*-test. The differences were considered significant when $P < 0.05$.

3. Results

The *in vitro* growth of *T. cruzi* epimastigotes was followed through 12 days while the growth of the parasites under the influence of the different concentrations of benzinidazole was followed through 3 days. There was a significant difference between the parasites concentrations when exposed to benzinidazole at 25 μmol/L, 50 μmol/L and 100 μmol/L ($P < 0.05$) (Figure 1).

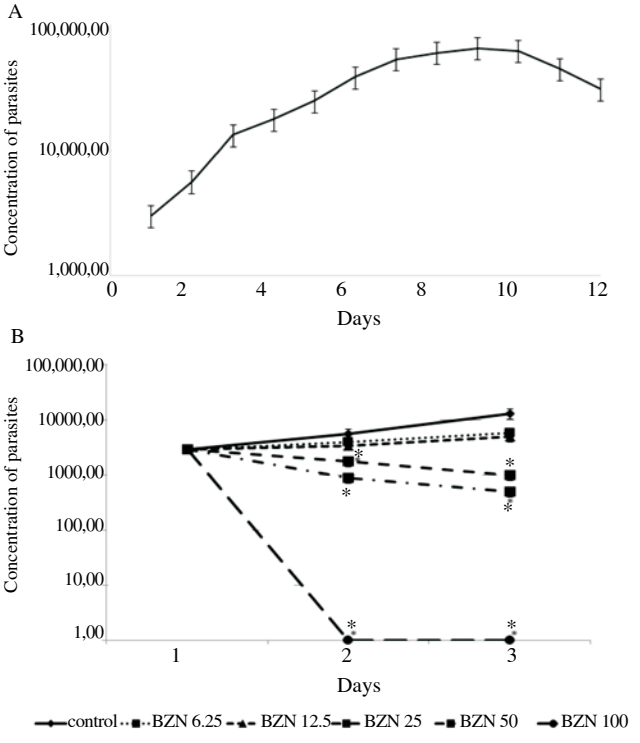

Figure 1. Growth curves of *T. cruzi* epimastigotes in LIT culture medium. A: Control growth curve accompanied through 12 days; B: Growth curve of parasites exposed to different concentrations of benznidazole accompanied through 3 days. *: $P < 0.05$ when compared to the control group.

The analysis of the biochemical profile of *T. cruzi* epimastigotes at 3, 6, 9 and 12 days of culture was performed to determine the

profile of the energetic metabolism of the parasite. The organic acids detected are detailed in Table 1.

Glucose was not used as an energy source for the epimastigotes form as its concentrations did not alter during the different culture days.

The anaerobic metabolism was only observed at the 3rd and 12th days of culture with a significant difference between the concentrations of lactate detected ($P < 0.05$).

There was a consumption of citrate and succinate from the culture medium as there was a significant difference between the concentrations detected in the culture medium and in the analysis from the parasites at the 6th, 9th and 12th days of culture ($P < 0.05$).

It was not possible to detect oxaloacetate and alpha-ketoglutarate. Also beta-hydroxybutyrate was only detected at the 12th day of culture.

The DMSO used to dilute the drugs did not influence the biochemical profile (Table 2). It is interesting to highlight that under the lowest concentrations of benznidazole (6.25 μmol/L and 12.5 μmol/L) the parasites showed a preference for the aerobic metabolism due to the detection of citrate, malate and succinate. When exposed to the highest concentrations of benznidazole (25 μmol/L, 50 μmol/L and 100 μmol/L) the parasites showed a preference for the anaerobic metabolism due to the detection of lactate. The concentrations of fumarate were not influenced by the drugs exposure (Table 2).

4. Discussion

The *in vitro* growth of *T. cruzi* epimastigotes in LIT cutlure medium has been extensively described in the literature[15-17]. Our findings are in accordance with such descriptions. The concentrations of parasites found in our study after the exposure to different concentrations of benzinidazole are in accordance with previous descriptions of *in vitro* survival of different strains of *T. cruzi* exposed to various concentrations of anti-parasitic drugs such as benzinidazole, nifurtimox and miltefosine[18]. Also Moreno *et al*.[19] have shown the *in vitro* susceptibility and resistance of different *T. cruzi* strains to benzinidazole. Other authors also used similar benzinidazole concentrations to determine *in vitro* trypanocidal effect on epimastigote of *T. cruzi*[20].

It is interesting to highlight that all the descriptions of the metabolic pathways of *T. cruzi* are in accordance to the organic acids detected in the 3rd day of culture[7]. However, the metabolic differences were not found in the literature when the parasite undergoes culture growth, and how different concentrations of benznidazole affect their metabolism is not found as well.

The non-detection of oxaloacetate may indicate that it was consumed for malate and fumarate production. The non-detection of

Table 1

Energetic metabolism of epimastigotes of *T. cruzi*, Y strain, at 3, 6, 9 and 12 days of culture.

Parameter	CM without parasites	3 Days	6 Days	9 Days	12 Days
Glucose (mg/dL)	353.25 ± 42.61	318.33 ± 40.56	299.00 ± 44.90	306.25 ± 16.97	286.25 ± 26.36
Pyruvate (μmol/L)	ND	ND	ND	0.12 ± 0.03	0.14 ± 0.04
Lactate (μmol/L)	ND	123.60 ± 53.61	ND	ND	17.07 ± 8.16**
Citrate (μmol/L)	26.98 ± 15.12	25.04 ± 18.42	1.38 ± 0.61*	6.38 ± 4.79*	3.82 ± 2.38*
Succinate (μmol/L)	373.38 ± 116.26	227.92 ± 240.21	8.09 ± 2.86*	12.40 ± 1.96*	24.25 ± 9.36*
Malate (μmol/L)	244.80 ± 159.96	915.10 ± 1193.96	ND	ND	ND
Acetate (μmol/L)	146.82 ± 22.69	45.58 ± 45.54	6.61 ± 3.84	ND	ND
Acetoacetate (μmol/L)	195.50 ± 110.55	90.73 ± 95.52	ND	ND	ND
BHBT (μmol/L)	ND	ND	ND	ND	2.17 ± 1.71
Propionate (μmol/L)	152.04 ± 80.72	41.40 ± 12.54*	ND	3.56 ± 0.83*	4.60 ± 1.83*
Fumarate (μmol/L)	3.89 ± 1.28	2.15 ± 1.28*	0.12 ± 0.05*	0.15 ± 0.03*	0.60 ± 0.87*
Urea (mg/dL)	14.42 ± 1.24	13.50 ± 2.43	16.60 ± 0.54*	17.40 ± 1.14*	17.00 ± 0.81*
Creatinin (mg/dL)	1.07 ± 0.14	0.88 ± 0.18	1.02 ± 0.04	1.00 ± 0.00	1.00 ± 0.07

Results are expressed as mean ± SD. CM: Culture medium; *: $P < 0.05$; ND: Non detected.

Table 2

Energetic metabolism of *T. cruzi* epimastigotes *in vitro* exposed to different concentrations of benznidazole.

Parameter	Parasites without drugs	DMSO control	Benznidazole				
			6.25 μmol/L	12.5 μmol/L	25 μmol/L	50 μmol/L	100 μmol/L
Glucose (mg/dL)	318.3 ± 42.61	322.83 ± 27.96	397.00 ± 3.60	351.33 ± 34.4	356.33 ± 2.08	355.33 ± 4.93	369.66 ± 4.93
Citrate (μmol/L)	29.77 ± 29.13	34.10 ± 13.72	14.85 ± 8.30	22.20 ± 20.22	ND	ND	ND
Malate (μmol/L)	915.10 ± 1193.96	427.16 ± 467.58	250.07 ± 107.64	561.22 ± 515.91	ND	ND	ND
Succinate (μmol/L)	227.92 ± 240.21	395.85 ± 186.70	251.73 ± 117.77	354.27 ± 317.34	ND	ND	ND
Lactate (μmol/L)	123.60 ± 53.61	332.58 ± 190.50	ND	ND	281.02 ± 150.42	442.35 ± 118.01	533.47 ± 84.82*
Acetoacetate (μmol/L)	90.73 ± 95.52	187.24 ± 60.04	ND	ND	ND	ND	ND
Fumarate (μmol/L)	2.15 ± 1.28	2.69 ± 1.24	1.36 ± 0.87*	2.85 ± 2.67	1.76 ± 0.61	2.99 ± 0.36	3.18 ± 0.28
Urea (mg/dL)	13.5 ± 2.49	13.50 ± 1.87	15.00 ± 0.10	13.66 ± 1.15	16.00 ± 0.12	16.00 ± 0.15	16.33 ± 0.57
Creatinin (mg/dL)	0.88 ± 0.18	0.90 ± 0.14	1.13 ± 0.05	0.96 ± 0.11	1.00 ± 0.10	1.03 ± 0.05	1.02 ± 0.04

Results are expressed as mean ± SD; *: $P < 0.05$ in comparison with the control group; ND: Non detected.

alpha-ketoglutarate indicates the preferential use of the glycosomal metabolic pathways for energy production due to the consumption of succinate to produce pyruvate in the epimastigotes of *T. cruzi*[7,21]. The detection of citrate indicates the presence of the mitochondrial metabolic pathway of energy production[7]. However, the decrease in the citrate, succinate and fumarate concentrations throughout the experimental days indicates that these pathways are being suppressed while the epimastigotes tend to undergo metacyclogenesis in the culture medium[22]. Also the consumption of the energetic sources from the culture medium throughout the experimental days may be detected by the decrease in the metabolic rates observed since the 6th day of culture.

The detection of pyruvate only at the 9th and 12th days of culture indicates that its secretion occurs as the final product of the pentose phosphate pathway described previously in *T. brucei*[23]. Phosphoenolpyruvate is the precursor of both pyruvate and acetate[24], and acetate was not detected in the 9th and 12th culture days; it is possible to infer that it was used to produce pyruvate in the stationary phase of the culture and to produce acetate in the logarithmic one. It is interesting to highlight that when there was the detection of pyruvate, acetate was not detected, and vice-versa, which means that the precursor of both these organic acids is consumed in one metabolic pathway or the other[24].

It is interesting to highlight that at the 3rd day of culture, when all the metabolites in the culture medium are in optimum concentration for the parasitary growth, all the metabolic pathways are active and the parasites present all the described final products of its energetic metabolism[7], including the anaerobic metabolism marker lactate. However, the anaerobic metabolic pathway is not observed in the 6th and 9th days of culture. Also the anaerobic metabolism was increased in the 12th day of culture due to the detection of lactate only. The lactate production is well described as a final product of the methylglyoxal detoxification pathway[25].

There was an increase in the proteins catabolism in the epimastigotes since the 6th day of culture due to the increase in the urea concentrations. The proteins catabolism is well described in *T. brucei* bloodstream forms[25].

The differences in secretion of acetate and pyruvate throughout the culture growth have been described previously by Shah-Simpson *et al*.[13]. Kim *et al*.[22] when analyzing the *in vitro T. brucei* metabolome detected that 29% of the produced metabolites were from the amino acids catabolism, one of which was acetate, while 13% of the metabolites were from the carbohydrate catabolism, one of which was pyruvate.

Hamedi *et al*.[16] using a metabolomic approach described that 20 μmol/L of benzinidazole has shown little effect on metabolism of epimastigotes for a 6-hour period, which is in accordance to our findings regarding the metabolic alterations of epimastigotes exposed to the lowest concentrations of benznidazole. As described in our study, the metabolic effect of benznidazole is better in concentrations higher than 25 μmol/L for at least 24 to 72 h of exposure.

Although one of the proposed mode of actions of benzinidazole is to be mediated via reduced intermediates which interfere in macromolecules such as lipids, DNA and proteins[16,26,27], the concentrations of benzinidazole used in our study did not interfere in the proteins catabolism due to the maintenance of urea, creatinin and fumarate levels after the exposure to the drugs. It is important to highlight that other factors that interfere in the protein turnover or in the translation inhibition were not evaluated.

On the other hand, the mitochondrial metabolites such as citrate and acetoacetate and the glycosomal ones such as succinate and malate were not detected after the exposure to high concentrations of benzinidazole. Considering that benzinidazole is a pro-drug that undergoes activation within the parasite's mitochondria, one or more of its metabolites may alter functions of these organelles or interfere within main enzymatic pathways[6,28]. Trochine *et al*.[6] described more than 14 metabolites in *T. cruzi* after benzinidazole exposure.

As Chagas disease is still a chronic disease in which the treatment is not entirely effective, Bermudez *et al*.[2] pointed out the need for improvements in the current treatment. The detection of metabolic responses of the parasite when exposed to the drug and therefore the identification of possible resistance or susceptibility[5,28] may help the development of more effective substances.

Therefore, due to the non-detection of the final products from neither the glycosome nor the mitochondrion, it is possible to conclude that one of the benznidazole modes of action is to impair the aerobic metabolism of the parasite inducing the anaerobic one due to the detection of lactate when exposed to higher concentrations of benznidazole. The metabolic analysis shown in this study may help the understanding of benzinidazole modes of action as well as to determine new drug targets.

Acknowledgment

This work was supported by the National Counsel of Technological and Scientific Development (CNPq) (Grant No. 302159/2016-9 and 471009/2013-0).

Conflict of interest statement

We declare that we have no conflict of interest.

References

[1] World Health Organization. Chagas disease (American trypanosomiasis). Geneva: World Health Organization; 2016. [Online] Available from: http://www.who.int/mediacentre/factsheets/fs340/en/ [Accessed on 28th April, 2016]

[2] Bermudez J, Davies C, Simonazzi A, Real JP, Palma S. Current drug therapy and pharmaceutical challenges for Chagas disease. *Acta Trop* 2016; **156**: 1-16.

[3] Pucar R, Moreno-Viguri E, Pérez-Silanes S. Challenges in Chagas

disease drug discovery: a review. *Curr Med Chem* 2016; **23**(28): 3154-70.

[4] Błaszczak-Świątkiewicz K, Mikiciuk-Olasik E. Some characteristics of activity of potential chemotherapeutics--benzimidazole derivatives. *Adv Med Sci* 2015; **60**(1): 125-32.

[5] Garavaglia PA, Lavarriere M, Cannata JJ, Garcia GA. Putative role of the aldo-keto reductase from *Trypanosoma cruzi* in benznidazole metabolism. *Antimicrob Agents Chemother* 2016; **60**: 2664-70.

[6] Trochine A, Creek DJ, Faral-Tello P, Barrett MP, Robello C. Benznidazole biotransformation and multiple targets in *Trypanosoma cruzi* revealed by metabolomics. *PLoS Negl Trop Dis* 2014; **8**: e2844.

[7] Maugeri DA, Cannata JJB, Cazzulo JJ. Glucose metabolism in *Trypanosoma cruzi*. *Essays Biochem* 2011; **51**: 15-30.

[8] Bauer S, Morris MT. Glycosome biogenesis in trypanosomes and the de novo dilemma. *PLoS Negl Trop Dis* 2017; **11**(4): e0005333.

[9] Bringaud F, Ebikeme C, Boshart M. Acetate and succinate production in amoebae, helminths, diplomonads, trichomonads and trypanosomatids: common and diverse metabolic strategies used by parasitic lower eukaryotes. *Parasitology* 2010; **137**: 1315-31.

[10] Seco-Hidalgo V, De Pablos LM, Osuna A. Transcriptional and phenotypical heterogeneity of *Trypanosoma cruzi* cell populations. *Open Biol* 2015; **5**: 150190.

[11] Shaw AK, Kalem MC, Zimmer SL. Mitochondrial gene expression is responsive to starvation stress and developmental transition in *Trypanosoma cruzi*. *mSphere* 2016; **1**(2): e00051-16.

[12] Vidal JC, Alcantara CL, de Souza W, Cunha-E-Silva NL. Loss of the cytostome-cytopharynx and endocytic ability are late events in *Trypanosoma cruzi* metacyclogenesis. *J Struct Biol* 2016; **196**: 319-28.

[13] Shah-Simpson S, Pereira CF, Dumoulin PC, Caradonna KL, Burleigh BA. Bioenergetic profiling of *Trypanosoma cruzi* life stages using seahorse extracellular flux technology. *Mol Biochem Parasitol* 2016; **208**(2): 91-5.

[14] Fraga CM, da Costa TL, de Castro AM, Reynoso-Ducoing O, Ambrosio J, Hernández-Campos A, et al. A benzimidazole derivative (RCB20) *in vitro* induces an activation of energetic pathways on *Taenia crassiceps* (ORF strain) cysticerci. *Exp Parasitol* 2017; **172**: 12-7.

[15] Nogueira NP, Saraiva FM, Sultano PE, Cunha PR, Laranja GA, Justo GA, et al. Proliferation and differentiation of *Trypanosoma cruzi* inside its vector have a new trigger: redox status. *PLoS One* 2015; **10**(2): e0116712.

[16] Hamedi A, Botelho L, Britto C, Fragoso SP, Umaki AC, Goldenberg S, et al. *In vitro* metacyclogenesis of *Trypanosoma cruzi* induced by starvation correlates with a transient adenylyl cyclase stimulation as well as with a constitutive upregulation of adenylyl cyclase expression. *Mol Biochem Parasitol* 2015; **200**(1-2): 9-18.

[17] Bourguignon SC, Mello CB, Santos DO, Gonzalez MS, Souto-Padron T. Biological aspects of the *Trypanosoma cruzi* (Dm28c clone) intermediate form, between epimastigote and trypomastigote, obtained in modified liver infusion tryptose (LIT) medium. *Acta Trop* 2006; **98**: 103-9.

[18] Botero A, Keatley S, Peacock C, Thompson RC. *In vitro* drug susceptibility of two strains of the wildlife trypanosome, *Trypanosoma copemani*: a comparison with *Trypanosoma cruzi*. *Int J Parasitol Drugs Drug Resist* 2017; **7**(1): 34-41.

[19] Moreno M, D'ávila DA, Silva MN, Galvão LMC, Macedo AM, Chiari E, et al. *Trypanosoma cruzi* benznidazole susceptibility *in vitro* does not predict the therapeutic outcome of human Chagas disease. *Mem Inst Oswaldo Cruz* 2010; **105**: 918-24.

[20] Kanesshima EN, Castro-Padro MAA, Toledo MJO, Araujo SM, Gomes ML. Trypanocidal activity of genotoxic concentration of benznidazole on epimastigote forms of *Trypanosoma cruzi*. *Acta Scientiarum* 2012; **34**: 321-7.

[21] Shameer S, Logan-Klumpler FJ, Vinson F, Cottret L, Merlet B, Achcar F, et al. TrypanoCyc: a community-led biochemical pathways database for *Trypanosoma brucei*. *Nucleic Acids Res* 2015; **43**: D637-44.

[22] Kim DH, Achcar F, Breitling R, Burgess KE, Barret MP. LC–MS-based absolute metabolite quantification: application to metabolic flux measurement in trypanosomes. *Metabolomics* 2015; **11**: 1721-32.

[23] Kerkhoven EJ, Achcar F, Alibu VP, Burchmore RJ, Gilbert IH, Trybilo M, et al. Handling uncertainty in dynamic models: the pentose phosphate pathway in *Trypanosoma brucei*. *PLoS Comput Biol* 2013; **9**: e1003371.

[24] Tielens L, Van Hellemond JJ. Differences in energy metabolism between Trypanosomatidae. *Parasitol Today* 1998; **14**: 265-71.

[25] Creek DJ, Mazet M, Achcar F, Anderson J, Kim DH, Kamour R, et al. Probing the metabolic network in bloodstream-form *Trypanosoma brucei* using untargeted metabolomics with stable isotope labelled glucose. *PLoS Pathog* 2015; **11**: e1004689.

[26] Rajão MA, Furtado C, Alves CL, Passos-Silva DG, de Moura MB, Schamber-Reis BL, et al. Unveiling benznidazole's mechanism of action through overexpression of DNA repair proteins in *Trypanosoma cruzi*. *Environ Mol Mutagen* 2014; **55**(4): 309-21.

[27] Maya JD, Cassels BK, Iturriaga-Vasquez P, Ferreira J, Faundez M, Galanti N, et al. Mode of action of natural and synthetic drugs against *Trypanosoma cruzi* and their interaction with the mammalian host. *Comp Biochem Physiol A Mol Integr Physiol* 2007; **146**: 601-20.

[28] Campos MCO, Leon LL, Taylor MC, Kelly JM. Benznidazole-resistance in *Trypanosoma cruzi*: evidence that distinct mechanisms can act in concert. *Mol Biochem Parasitol* 2014; **193**: 17-9.