A novel ABCG-like transporter of *Trypanosoma cruzi* is involved in natural resistance to benznidazole

Bianca Zingales[1]/+, Rafael Gomes Aquino Araujo[1], Margoth Moreno[1], Jaques Franco[1], Pedro Henrique Nascimento Aguiar[2], Solange Lessa Nunes[1], Marcelo Nunes Silva[1], Susan Ienne[1], Carlos Renato Machado[2], Adeilton Brandão[3]

[1]Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, São Paulo, SP, Brasil
[2]Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brasil
[3]Laboratório Interdisciplinar de Pesquisas Médicas, Instituto Oswaldo Cruz-Fiocruz, Rio de Janeiro, RJ, Brasil

Benznidazole (BZ) is one of the two drugs used for Chagas disease treatment. Nevertheless therapeutic failures of BZ have been reported, which were mostly attributed to variable drug susceptibility among Trypanosoma cruzi *strains. ATP-binding cassette (ABC) transporters are involved in a variety of translocation processes and some members have been implicated in drug resistance. Here we report the characterisation of the first* T. cruzi *ABCG transporter gene, named* TcABCG1*, which is over-expressed in parasite strains naturally resistant to BZ. Comparison of* TcABCG1 *gene sequence of two TcI BZ-resistant strains with CL Brener BZ-susceptible strain showed several single nucleotide polymorphisms, which determined 11 amino acid changes. CL Brener transfected with TcI transporter genes showed 40-47% increased resistance to BZ, whereas no statistical significant increment in drug resistance was observed when CL Brener was transfected with the homologous gene. Only in the parasites transfected with TcI genes there was 2-2.6-fold increased abundance of* TcABCG1 *transporter protein. The analysis in wild type strains also suggests that the level of* TcABCG1 *transporter is related to BZ natural resistance. The characteristics of untranslated regions of* TcABCG1 *genes of BZ-susceptible and resistant strains were investigated by computational tools.*

Key words: Chagas disease - benznidazole resistance - ABCG transporter - *T. cruzi* strains - transcript abundance - single nucleotide polymorphisms

The protozoan *Trypanosoma cruzi* is the etiological agent of Chagas disease, which ranks among the world's most neglected diseases. According to estimates by the Pan American Health Organization and the World Health Organization (WHO), 7.7-10 million people are chronically infected with *T. cruzi* and 10,000-14,000 deaths per year are caused by Chagas disease (Moncayo & Silveira 2009, WHO 2012). Only two drugs are available for Chagas disease treatment, benznidazole (BZ) and nifurtimox (NF). Both drugs are nitroheterocyclic compounds which frequently cause severe side effects and have limited efficacy in the chronic phase of the disease (Rassi Jr et al. 2012). The reasons for treatment failures are unknown, but may be due to variable drug susceptibility among *T. cruzi* strains, characteristics of the host's immune system and/or unfavourable drug pharmacokinetic properties (Urbina 2010). Nevertheless, several nonrandomised clinical studies report the efficacy of the etiological treatment to reduce the progression of chronic Chagas heart disease (Sosa-Estani et al. 2009).

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+ Corresponding author: bszodnas@iq.usp.br
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Although in recent years novel chemical classes have shown promising activity against *T. cruzi*, no new drug is under development (Zingales et al. 2014). Therefore, efforts should be devoted to elucidating mechanism(s) underlying BZ and/or NF natural resistance.

Experimental evidence from several laboratories has shown that BZ and NF exhibit divergent activities against different *T. cruzi* strains (Filardi & Brener 1987, Murta et al. 1998, Villarreal et al. 2004, Moreno et al. 2010). Such differences most certainly are related to the high phenotypic and genotypic diversity among *T. cruzi* strains. Currently the parasite strains are classified into six discrete typing units (DTUs), designated as TcI-TcVI (Zingales et al. 2009). The rationale for this classification, DTU eco-epidemiological features and correlation with natural and experimental infection has been reviewed (Miles et al. 2009, Zingales et al. 2012).

Specific ATP-binding cassette (ABC) transporters have been implicated in drug resistance in protozoan parasites such as *Leishmania*, *Trypanosoma* and *Plasmodium* (Klokouzas et al. 2003, Leprohon et al. 2006, Sauvage et al. 2009). ABC transporters constitute one of the largest families of membrane proteins that mediate the ATP-driven unidirectional transport of a variety of molecules across biological membranes (Higgins 1992). Substrates include lipids, amino acids, peptides, toxins and chemotherapeutic drugs. ABC transporters have been associated to the development of resistance of tumours to anticancer drugs, as well as antibiotic resistance in pathogenic microorganisms (Klokouzas et al. 2003, Higgins 2007, Sharom 2008, Sauvage et al. 2009).

In general, ABC transporters are composed of two hydrophobic transmembrane domains (TMDs), each usually with six alpha-helical transmembrane segments, and two cytoplasmic nucleotide-binding domains (NBDs). The NBDs contain three conserved sequence elements: the Walker A and Walker B motifs and the ABC signature sequence, which is characteristic of ABC proteins (Higgins 1992). The TMDs form the putative pathway for substrates across the lipid bilayer and are believed to determine the substrate specificity of the transporter (Velamakanni et al. 2007). ABC "full-size" transporters, symbolised as TMD-NBD_2, are mainly localised in the plasma membrane. Numerous ABC proteins, called "half-size" transporters, are composed of a single NBD fused to a single TMD. According to the N or C-terminal location of these domains, they are represented as TMD-NBD or NBD-TMD. The "half-size" transporters undergo homo or heterodimerisation for their physiological activity (Velamakanni et al. 2007). Eukaryotic ABC proteins are divided into eight families (ABCA-ABCH) (Igarashi et al. 2004). Representatives of the ABCA, ABCB and ABCC ("full-size" transporters) and ABCD and ABCG ("half-size" transporters) families have been described in protozoan parasites [reviewed by Klokouzas et al. (2003), Leprohon et al. (2006) and Sauvage et al. (2009)].

In the present paper we report the identification of one ABC transporter gene of the G family, named *TcABCG1,* which is over-expressed in parasite strains naturally resistant to BZ. Transfection of the *TcABCG1* gene of two BZ-resistant strains in CL Brener BZ-susceptible epimastigotes increased by 40-47% the 50% inhibitory concentration (IC_{50}) value to BZ in the recipient parasites. The relative abundance of *Tc*ABCG1 transcripts and the level of the transporter protein were determined in the transfected cultures. The data suggest that *Tc*ABCG1 transporter is one of the elements involved in *T. cruzi* resistance to BZ.

MATERIALS AND METHODS

Parasite strains and cultivation - The characteristics of the *T. cruzi* strains employed in this study are summarised in Table I. Epimastigote forms were cultured in liver infusion-tryptose medium supplemented with 10% foetal calf serum at 28ºC.

Primers - The sequence and characteristics of the primers are described in Supplementary Table I.

Assay to evaluate BZ susceptibility - The assay was performed as previously reported (Moreno et al. 2010), with exponentially growing epimastigotes. Three independent assays with three replicates in each assay were performed on separate days. For calculation of IC_{50}, corresponding to the drug concentration that inhibited parasite growth by 50%, the data were treated with SigmaPlot v.11 software (Systat Software Inc), employing the four-parameter logistic equation.

DNA microarray experiments - DNA microarray slides, kindly provided by the Pathogen Functional Genomics Resource Center, bearing replicates of 70-mer oligonucleotides representative of 12,288 CL Brener open reading frames (ORFs) (pfgrc.jcvi.org/index.php/microarray/array_description/trypanosoma_cruzi/version1.html) were employed. Total parasite RNA was extracted with Trizol reagent (Life Technologies) and DNase-treated according to standard procedures. Approximately 20 µg RNA was labelled with Cy3 or Cy5-dCTP for the first-strand cDNA synthesis (Baptista et al. 2004). Hybridisation conditions, image acquisition and data analysis were as previously described (Baptista et al. 2004, 2006). Data were obtained for two biological replicates (independent parasite harvests). For each replicate a dye-swap experiment was performed.

Real time reverse transcription-polymerase chain reaction (RT-PCR) - RT of 5 µg DNAse-treated total RNA was carried out with the SuperScript First-Strand Synthesis System (Invitrogen) and Oligo$(dT)_{12-18}$ primers, according to the manufacturer's instructions. The levels of *TcABCG1* transcripts were determined by real-time PCR using the product of cDNA synthesis reactions as template and ABC.F and ABC.R primers for the *TcABCG1* gene. Real-time PCR was performed in the Mas-

TABLE I

Characteristics of *Trypanosoma cruzi* strains

Strain	DTU[a]	Host/vector	IC_{50} BZ (µM) (mean ± SD)[b]	BZ phenotype
Silvio X10 cl1	TcI	*Homo sapiens*	26.1 ± 2.5	R
YuYu	TcI	*Triatoma infestans*	40.5 ± 1.8	R
Colombiana	TcI	*H. sapiens*	34.1 ± 0.8	R
Berenice 62	TcII	*H. sapiens*	14.6 ± 3.6	S
VL 10	TcII	*H. sapiens*	30.4 ± 2.9	R
Esmeraldo cl3	TcII	*H. sapiens*	26.7 ± 0.3	R
115	TcV	*H. sapiens*	7.6 ± 1.6	S
CL Brener	TcVI	*T. infestans*	13.2 ± 0.8	S

a: discrete typing units (DTUs) classified according to Zingales et al. (2009); *b*: benznidazole (BZ) sensitivity in epimastigotes; IC_{50}: 50% inhibitory concentration; R: resistant; S: susceptible; SD: standard deviation.

tercycler Realplex apparatus (Eppendorf) using SYBR® Green PCR Master Mix (Applied Biosystems). The relative amount of PCR products was determined based on the threshold cycle value and amplification efficiencies and was normalised by dividing the values by the relative amount of the glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene (XM_814806) used as calibrator and amplified with the pair of primers GAPDH.F and GAPDH.R. All the samples were tested in triplicates in two independent experiments.

Cloning and sequencing of TcABCG1 gene - *TcABCG1* gene of the YuYu strain was PCR amplified from total DNA with the pair of primers *Tc*ABCG1.For and *Tc*ABCG1.Rev flanking the gene ORF. Amplicons were cloned into pGEM®-T easy vector (Promega). At least six plasmid clones were sequenced. Chromatograms were analysed with Phred-Phrap-Consed software (Phred scores > 80) (Gordon et al. 1998). The processed sequences were clustered generating the consensus sequence. Nucleotide and predicted amino acid sequences were aligned using CLUSTALX 2.0.12 and alignments were manually adjusted using BioEdit.

Transfection studies - The complete *TcABCG1* ORF of CL Brener, Silvio and YuYu strains was PCR-amplified from genomic DNA with the proofreading *Pfu* DNA polymerase (Fermentas) and the pair of primers *Tc*ABCG1.For and *Tc*ABCG1.Rev. Amplicons were cloned in pGEM-T Easy vector (Promega) and the recombinant plasmids were recovered by alkaline lysis using the Wizard Plus SV Miniprep kit (Promega). The identity of the cloned genes was confirmed by sequencing. The expression vector pROCKNeo (DaRocha et al. 2004) was used in the transfection studies. This vector allows the integration of the foreign gene in the β-tubulin *locus* of *T. cruzi* (DaRocha et al. 2004). *TcABCG1* gene cloned in pGEM-T Easy was released from the vector by digestion with *Xba*I and *Xho*I enzymes and ligated to pROCKNeo digested with the same enzymes. CL Brener epimastigotes were transfected by electroporation with 50 µg linearised plasmid DNA digested with *Not*I as previously described (DaRocha et al. 2004). Transfected parasites were selected after eight weeks culturing in the presence of 200 µg/mL neomycin.

Southern blot - Genomic DNA was digested with *Bam*HI, separated on 0.8% agarose and analysed by probing with α-^{32}P radiolabelled probes. A 616-base pairs (bp) DNA fragment localised between nt 911-1527 of the *TcABCG1* gene was independently amplified from CL Brener and Silvio genomic DNA with the pair of primers *Tc*ABCG616F and *Tc*ABCG616R. A 435-bp probe for the neomycin resistance (*NeoR*) gene was amplified from pROCKNeo DNA with the pair of primers Neo-F and Neo-R. Blotting and generation of radioactive DNA probe fragments by random primed labelling were performed as described (Baptista et al. 2006).

Antibodies to TcABCG1 and immunoblotting - A region comprised between nt 232-960 of the *TcABCG1* gene was amplified by PCR with PQE2.F and PQE2.R primers and cloned in the expression vector pQE-30 Xa (Quiagen). After transformation of *Escherichia coli* XL1 Blue MR, standard procedures were followed for the induction and purification of the ~26 kDa recombinant protein. Antibodies were obtained in male Balb/c mice following immunisation with the recombinant protein by intraperitoneal route. Western blot was performed with total protein extract (2 x 10^7 cells/lane) resolved on 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis. Proteins were electrotransferred to midi nitrocellulose membranes with a Trans-Blot Turbo Transfer Starter System (BioRad) and incubated with the antibodies at 4ºC for 16 h. After washing, bound antibodies were detected with the secondary antibody goat anti-mouse IgG, conjugated to IRDye 800 CW (Li-COR, GmbH, Germany) at the dilution 1:15,000 in tris buffered saline-Tween 0.05% for 1 h at room temperature. To assess variations in protein loading, the membrane was incubated with a polyclonal antibody to *T. cruzi* glycosomal GAPDH (gGAPDH) and processed with a second antibody goat anti-rabbit IgG as above. The images were obtained with LiCOR Odyssey Infared Scanner and analysed with Image Studio 4.0 software.

In silico analysis of the 5' and 3' untranslated regions (UTR) of TcABCG1 genes - To estimate the position of the 5'UTR, we used an approach that takes into account two parameters: (i) the length of 5'UTR for any *T. cruzi* gene does not exceed 20% of the respective ORF length (Brandão & Jiang 2009) and (ii) the relative position of poly-pyrimidine tracts preceding a dinucleotide AG (possible trans-splicing site) in a segment upstream of initiation codon (Campos et al. 2008). We estimated 3'UTRs based upon previous work of Brandão and Jiang (2009), who pointed out that 3'UTR is on average 3-3.5 x longer than 5'UTR. For the sequences of the genes of Esmeraldo, Silvio, CL Brener NEsmo and Esmo haplotypes, we estimated a 5'UTR length of ~130 bases (without SL) and approximately 400 bases for 3'UTR. These estimates are supported by transcript sequences that were retrieved from the Sequence Read Archive (*SRA*) section of National Center for Biotechnology Information (transcriptome reads). We found short transcript sequences that partially map to the extremities of both UTR. The SRA reads accessions are 5' UTR - SRR799814.5318050, SRR1118380.5167076 and 3' UTR - SRR1118380.6178627, SRR1118380.7236671, SRR1118380.5954204, SRR1118380.3343870.

The UTR sequences - Approximately 160 bases for 5'UTR (including 39 nt of SL) and 400 bases for 3'UTR were submitted to ProbablePair algorithm in the RNAstructure Web Server (rna.urmc.rochester.edu/RNAstructureWeb/) (Reuter & Mathews 2010). The predicted bp were selected at 97% threshold for 5'UTR and 95% for 3'UTR. These were the highest values upon which the bp arise in all sequences in the analysed strains. To plot the predicted bp arc diagrams, we used the program R-chie (e-rna.org/r-chie/) (Lai et al. 2012). In order to evaluate the presence of conserved or potentially active motifs in the 3'UTR, the segments containing predicted bp were used to both searching an UTR database and scanning

by RNA motifs finder programs. The databases and motifs finder programs are described below. Searching and scanning were carried out under the parameters suggested by the software developer: (i) UTR database (UTRdb) (utrdb.ba.itb.cnr.it/), (ii) CMfinder 2.0 web server (wingless.cs.washington.edu/htbin-post/unrestricted/CMfinderWeb/CMfinderInput.pl) (Yao et al. 2006), (iii) MEME software (meme.sdsc.edu) (Bailey et al. 2009).

Statistics - The statistical analysis regarding differences in transcript abundance of *TcABCG1* gene between BZ-susceptible and BZ-resistant strains was performed with the Student's t test. The test was applied with a confidence interval (CI) of 95% (p = 0.05) to accept the null hypothesis (H_0) (there is no significant difference in the transcript levels between the 2 groups of strains). One-way ANOVA followed by Holm-Sidak multiple comparisons method was employed to analyse the differences of BZ sensitivity between the groups of transfected parasites vs. a control group. Overall significance level = 0.05. All the data were analysed with SigmaPlot v.11.0.

RESULTS

Variability of BZ sensitivity among T. cruzi strains - The sensitivity to BZ was determined in epimastigote forms of eight strains belonging to four DTUs (TcI, TcII, TcV and TcVI) (Table I). These DTUs are predominant in Chagas disease patients in different regions of Latin America (Miles et al. 2009, Zingales et al. 2012). We observed up to five-fold variation of the IC_{50} values to BZ among the strains (115 strain, 7.6 ± 1.6 µM; YuYu strain, 40.5 ± 1.8 µM). The rationale for considering a strain susceptible or resistant to BZ has been discussed elsewhere (Moreno et al. 2010) and was based on the agreement between the drug activity in vitro and in vivo (Filardi & Brener 1987).

One ABC transporter is over-expressed in BZ-resistant strains - The differential gene expression between BZ-resistant and BZ-susceptible strains was initially investigated by the hybridisation of CL Brener DNA microarray slides with epimastigote cDNAs of the VL10 (IC_{50} 30.4 ± 2.9 µM) and the 115 (IC_{50} 7.6 ± 1.6 µM) strains. These strains were chosen since both were isolated from patients in the chronic phase of Chagas disease in the state of Minas Gerais, Brazil. Analysis of the hybridisation data indicated that 578 probes were up-regulated in the VL10 strain and 676 probes up-regulated in the 115 strain. The list describing these probes will be published elsewhere (M Moreno et al., unpublished observations). Among the probes up-regulated in VL10 there were two oligonucleotides, QTC00005_L_15 and QTC00014_D_3, showing cDNA hybridisation ratios VL10/115 1.8 and 3.6, respectively. These probes (ftp.jcvi.org/pub/data/PFGRC/MAIN/microarray/annotation/T_cruzi/version1/T_cruzi_1_CLBrener.txt) represent different regions of the same putative ABC transporter gene of CL Brener non-Esmeraldo-like haplotype (GenBank XM_813521.1). It should be pointed out that oligonucleotides representing the Esmeraldo-like haplotype of the same gene (GenBank XM_801573.1) were not spotted on the microarray slide. As described by El-Sayed et al. (2005), CL Brener is a hybrid strain and the two haplotypes, corresponding to the two ancestral genomes, were named Esmeraldo-like and non-Esmeraldo-like. Here, the two haplotypes will be abbreviated as Esmo and NEsmo, respectively.

The identified ABC transporter gene (1,998 bp) is a single copy gene located on chromosome 37 of CL Brener (Weatherly et al. 2009). The sequence of the transporter protein (665 residues), encoded by the NEsmo haplotype (XP_818614), and the secondary structure, predicted with the SOSUI system (harrier.nagahama-i-bio.ac.jp/sosui/), are shown in Fig. 1. According to this scheme (Fig. 1B), the NBD would be comprised between residues 1-~400. The ATP-binding site, spanning from residues 74-296, contains the Walker A, ABC signature and Walker B motifs. The six presumed TMD α-helices are indicated in the Fig. 1. Based on these features, we concluded that the transporter belongs to the half-size ABCG family (NBD-TMD structure arrangement). Being the first ABCG transporter to be characterised in *T. cruzi* it was named *Tc*ABCG1.

To validate the differential gene expression, the relative transcript abundance of *TcABCG1* was determined by real time RT-PCR in three BZ-susceptible and five BZ-resistant strains (Fig. 2). CL Brener was used as the reference organism (relative abundance 1). To verify if the differences in transcript abundance of *TcABCG1* between the two groups of strains (BZ-susceptible and BZ-resistant) were statistically significant, the data were submitted to the Student's *t* test. The test was applied with a 95% CI (p = 0.05) to accept the H_0. The analysis indicated that the separation between the two groups was statistically significant (t = -2.935), allowing us to conclude that the BZ-resistant strains had higher levels of *TcABCG1* transcripts.

*Variation of Tc*ABCG1 *protein sequence in T. cruzi strains* - Next we verified whether, besides differences in the level of expression of *TcABCG1* gene among the strains, there would be also sequence variations. The sequence of the *TcABCG1* transporter of the Silvio strain is available (GenBank EKG04318.1). Then we sequenced the *TcABCG1* gene of the YuYu strain (GenBank KM520157), which is more resistant to BZ than Silvio and also belongs to DTU TcI. Sequence alignment of the two genes showed six single nucleotide polymorphisms (SNPs) (Supplementary Table II), but the ABCG proteins were identical (Table II).

Alignment of the predicted TcI proteins with the protein encoded by the NEsmo haplotype indicated 11 amino acid residue changes (Table II), whose localisation is indicated in Fig. 1B. Three variations were verified before the putative ATP-binding site. In this site, Ser80 of NEsmo was substituted by Ala in TcI strains. As expected, no variation occurred in the Walker A and B motifs or the ABC signature. Three amino acid changes were observed after the ATP binding site: Gly375, Arg376 and Met394 of NEsmo were replaced, respectively, by Ser, Met and Ile in Silvio and YuYu strains. The differences in the charge of the lateral chains of the amino acid residues may introduce some structural modifications. In the TMD, which seems to confer specificity to the

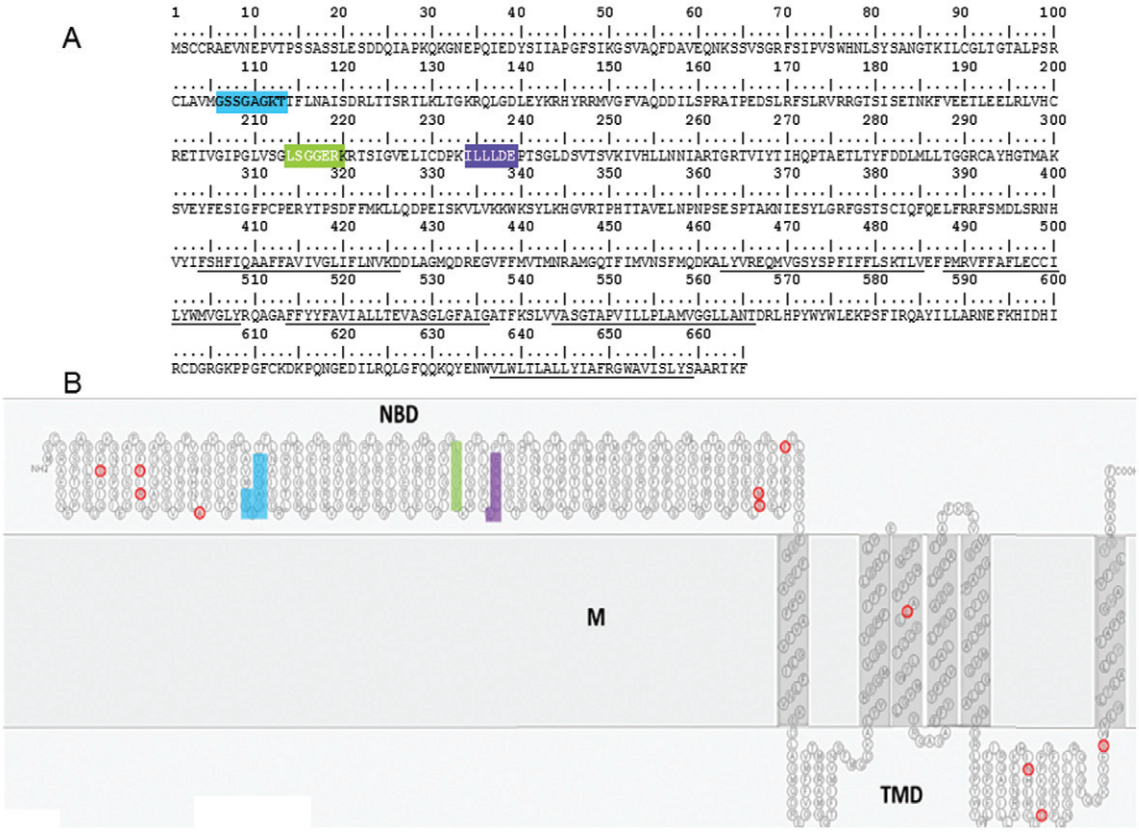

Fig. 1: protein sequence of *Tc*ABCG1 transporter (665 residues) encoded by CL Brener NEsmo haplotype. Rectangle blue, Walker A motif, green, ABC signature and purple, Walker B motif. The putative localisation of the six α-helices is underlined; B: secondary structure of *Tc*ABCG1 predicted with the SOSUI system (harrier.nagahama-i-bio.ac.jp/sosui/). The localisation of nucleotide binding domain (NBD), membrane (M) and transmembrane domain (TMD) is indicated. Within the NBD, Walker A motif, ABC signature and Walker B motif are indicated as above. In the TMD, the six α-helices are shown in grey. Red circles denote the amino acid residues that differ between TcI strains and NEsmo (Table II).

molecule to be translocated (Velamakanni et al. 2007), no amino acid changes occurred in five of the six putative α-helices (Fig. 1B). In the third α-helix, Phe495 was replaced by Leu. This is a conservative change of hydrophobic amino acids and unlikely to have a major effect on function. Three amino acid substitutions were observed in the region between the fifth-sixth α-helices: Asp598, Gly604 and Asn635 of NEsmo, substituted, respectively, by Asn, Asp and Ser in TcI strains.

Participation of TcABCG1 transporter in BZ natural resistance - To investigate the possible influence of *Tc*ABCG1 transporter structure in the sensitivity to BZ, we transfected the genes of the two TcI BZ-resistant strains (Silvio and YuYu) and the NEsmo haplotype in BZ-susceptible CL Brener epimastigotes. The genes were cloned in pROCK.Neo plasmid (Fig. 3A), which can be integrated in any of *T. cruzi* multiple β-tubulin *loci* (DaRocha et al. 2004). As controls the parasites were also transfected with the empty vector or PBS. The transfected parasites were submitted to drug selection with 200 µg/mL G418 for eight weeks, when all the parasites electroporated with PBS died.

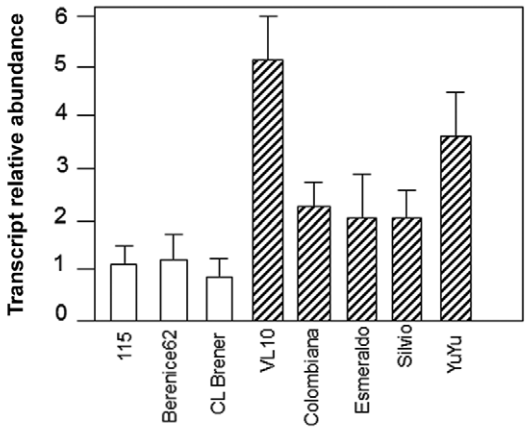

Fig. 2: relative transcript abundance of *TcABCG1* transporter in epimastigote forms of benznidazole (BZ)-susceptible (white rectangles) and BZ-resistant (hatched rectangles) strains. The data represent the mean and standard deviation of two biological replicates with triplicates in each assay. CL Brener (NEsmo haplotype) was used as reference (relative abundance 1).

TABLE II

Variation of amino acid residues in the *Tc*ABCG1 transporter[a]

Strains	GenBank	28	48	50	80	124	375	376	382	394	462	495	507	598	604	611	635	637
Silvio	EKG0431	S	F	R	A	T	S	M	C	I	A	L	L	N	D	F	S	V
YuYu	KM52015	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
CLB.NEsmo	XP_818614.1	P	S	K	S	.	G	R	.	M	.	F	.	D	G	.	N	.
CLB.Esmo	XP_806666.1	.	S	K	.	K	.	R	G	.	P	F	F	D	G	Y	.	I
		NBD[b]									TMD[b]							

a: the amino acid position refers to the protein sequence of Silvio X10 cl1 strain; *b*: position of nucleotide binding domain (NBD) and transmembrane domain (TMD) inferred with the SOSUI system (Fig. 1B). The dots represent the same amino acid residues as Silvio.

The integration of the exogenous *TcABCG1* genes in CL Brener genome was investigated by Southern blot. Total DNA of CL Brener wild type (WT), Silvio WT and transfectants was digested with *Bam*HI and separated in an agarose gel (Fig. 3B). The blot was hybridised to a probe represented by the mixture of a 616-bp DNA fragment of the *TcABCG1* gene of CL Brener and Silvio strains (Fig. 3C). This procedure was necessary since there are differences in the nucleotide sequence of this region between the two strains. The probe identified a high molecular mass band (~21 kb) in the WT strains and CL Brener transfected with the empty plasmid. In *TcABCG1* transgenes a doublet was verified around 21 kb: one corresponding to the CL Brener endogenous gene and the other, most probably, to the introduced gene. Two additional bands were also seen, one of molecular mass higher than 21 kb and another of ~7 kb.

Hybridisation of *Bam*HI-digested DNA (Fig. 3D) with a probe derived from pROCK.Neo gene that confers *NeoR*, confirmed the integration of the vector in the parasite genomes (Fig. 3E). In the transfectants bearing the transporter gene, three bands were identified (Fig. 3E) of the same size as the bands recognised by the *TcABCG1* probe in the transgenes (Fig. 3C). As expected, the neomycin probe did not hybridise with CL Brener WT DNA. A weak band of molecular mass ~21 kb was observed in CL Brener transfected with the empty pROCK vector.

T. cruzi genes encoding α and β-tubulin are arranged in a cluster with an alternating α and β-array with a basic repeat unit length of 4.3 Kb (Maingon et al. 1988). In addition, other pairs of alternating α and β-tubulin sequences appear to be physically separated from the basic group (Maingon et al. 1988). Each β-tubulin gene has one *Bam*HI restriction site. The three bands observed in the transgenic parasites hybridising with *TcABCG1* and neomycin probes are the result of the insertion of pROCK.Neo in one of the many β-tubulin copies. As a whole, the Southern blot data confirmed that the exogenous *TcABCG1* genes were integrated in CL Brener genome, most probably in the same *locus*.

BZ susceptibility in the transfected parasites and CL Brener WT was determined. Data in Fig. 4A refer to the IC_{50} values (mean ± standard deviation). In relation to CL Brener WT, we verified 47% and 41% increase of the IC_{50} values in the cultures transfected with the *TcABCG1* gene of

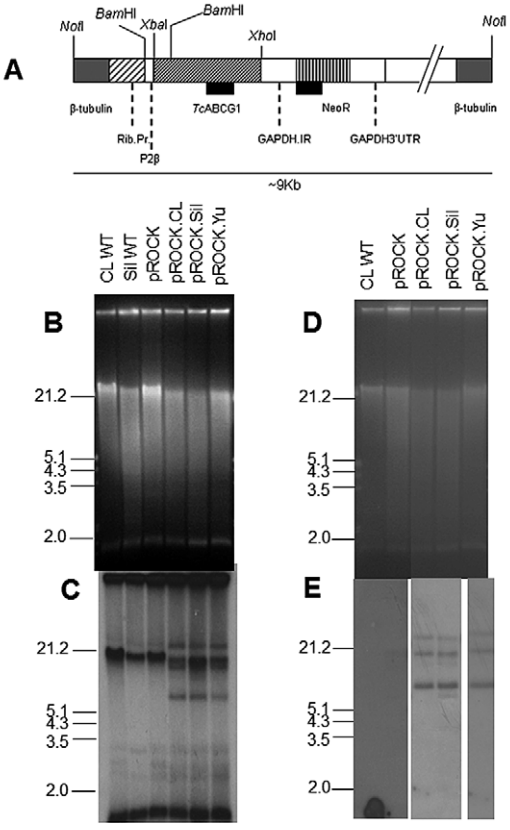

Fig. 3A: schematic representation of the pROCK.Neo *TcABCG1* vector construct. The β-tubulin *locus* is followed by the *Trypanosoma cruzi* ribosomal promoter and the P2β 5' intergenic region for spliced leader addition. The open reading frame of *TcABCG1* gene was cloned between the *Xba*I and *Xho*I unique restriction sites, followed by the 3' untranslated region plus intergenic region derived from the glyceraldehyde 3-phosphate dehydrogenase I gene. The localisation of the neomycin resistance (*NeoR*) gene used as a drug-selectable marker is indicated. Black rectangles denote the localisations of the 616-base pair (bp) probe of the *TcABCG1* gene and the 435-bp probe of the *NeoR* gene, both used in the Southern blot. The unique restriction site for the enzyme *Not*I was used for vector linearisation; B-E: Southern blot of total DNA digested with *Bam*HI. Ethidium bromide stained gels (B, D) and hybridisations with ^{32}P-labelled probes 616-bp DNA fragment of the *TcABCG1* gene (C) and *NeoR* pROCK gene (E); E: the membrane strips corresponding to CL Brener wild type (WT), empty pROCK and pROCK.Yu were exposed for longer times since the hybridisation signal. Molecular mass markers are indicated in kb.

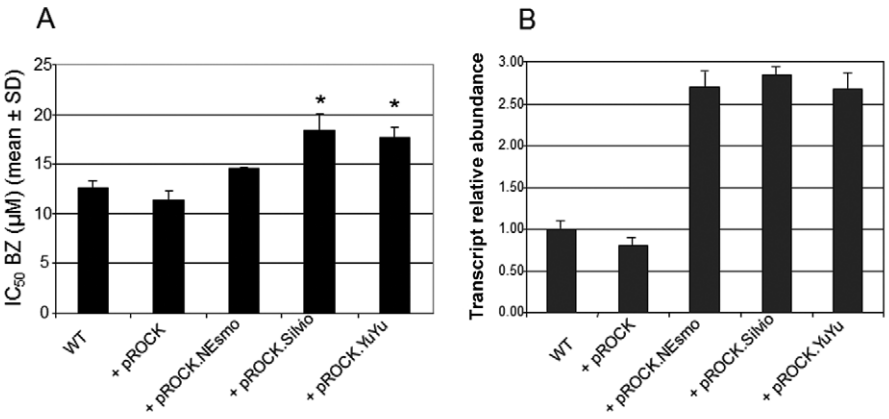

Fig. 4: susceptibility to benznidazole (BZ) (A) and transcript relative abundance (B) in CL Brener epimastigotes transfected with the *TcABCG1* gene of CL Brener NEsmo, Silvio and YuYu strains cloned in pROCK.Neo vector. The parameters were also obtained for CL Brener wild type (WT) and parasites transfected with the empty vector. Mean and standard deviation (SD) are indicated and were obtained from at least three independent assays (biological replicates) with three replicates in each assay. Asterisks mean significant values compared to CL Brener WT. IC_{50}: 50% inhibitory concentration.

TABLE III

One-way ANOVA followed by Holm-Sidak multiple comparisons method was used to analyse the differences in the mean values of benznidazole sensitivity between the transfected cultures and CL Brener wild type (WT) (Fig. 3)

Comparison	Difference of means	t	Unadjusted p	Critical level	Significant?[a]
CL WT vs. empty pROCK	1.221	1.644	0.121	0.050	No
CL WT vs. pROCK.CL	2.007	2.482	0.025	0.025	No
CL WT vs. pROCK.Silvio	5.917	7.965	< 0.001	0.013	Yes
CL WT vs. pROCK.YuYu	5.167	6.955	< 0.001	0.017	Yes

a: there is a statistically significant difference when $p \leq 0.001$; overall significance level: 0.05.

Silvio and YuYu strains, respectively. In the culture transfected with CL Brener gene the IC_{50} increase was 16%.

The one-way ANOVA followed by Holm-Sidak multiple comparisons method were employed to analyse the differences in the mean values of BZ sensitivity (IC_{50} values) between the transfected cultures and CL Brener WT (control group) (Table III). We concluded that the differences in the mean IC_{50} values of the parasites transfected with the Silvio and YuYu genes are greater than would be expected by chance ($p \leq 0.001$). On the other hand, the increase of the resistance to the drug in parasites transfected with the homologous NEsmo haplotype or the empty vector had no statistical significance ($p = 0.025$ and 0.121, respectively).

Relative abundance of TcABCG1 transcripts and transporter protein in CL Brener transfected parasites - The relative abundance of the *TcABCG1* transcripts in the transfected parasites was determined by real time RT-PCR (Fig. 4B). An ~2.7-fold increase was verified in the parasites transfected with pROCK recombinant vectors. No significant statistical difference was verified regarding the strain source of the *TcABCG1* gene: CL Brener, Silvio or YuYu (ANOVA test $p < 0.05$).

To estimate the levels of *Tc*ABCG1 transporter protein, we obtained an antiserum to a protein region comprised between the amino acid residues 77 and 320. This region (~26 kDa) contains the Walker A and B motifs and the ABC signature. The immunoblot of total protein of CL Brener WT and transfected parasites incubated with the anti-*Tc*ABCG1 serum showed the recognition of an ~76 kDa band, corresponding to the *Tc*ABCG1 protein (Fig. 5A). To correct for differences in signal intensities due to variations in the amount of protein loaded in each lane, the blot was incubated with an antiserum to *T. cruzi* gGAPDH. Densitometric quantifications of the signals allowed us to conclude 2-2.6-fold increase of the *Tc*ABCG1 protein in the cultures electroporated with the TcI gene of Silvio and YuYu strains, respectively (Fig. 5A). In CL Brener transfected with the NEsmo haplotype an increase of only 1.1 fold was observed.

Transporter abundance in natural BZ-resistant and susceptible strains - The anti-*Tc*ABCG1 serum was

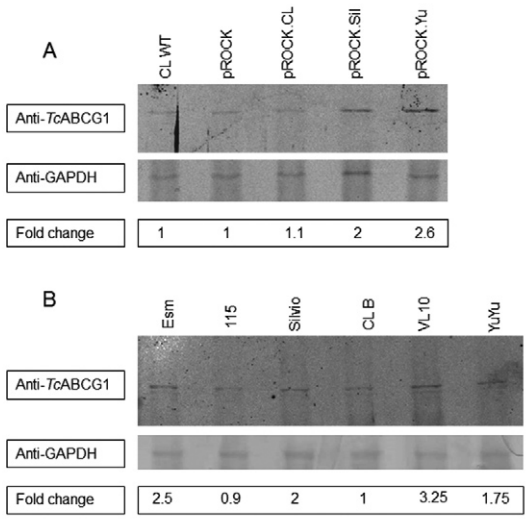

Fig. 5: western blot with total protein of *T. cruzi* epimastigotes. A: CL Brener wild type (WT) and CL Brener pROCK-transfected parasites; B: WT strains. In each panel, membranes were incubated with an antiserum against a region of the *Tc*ABCG1 transporter. Subsequently, the blot was incubated with an antiserum to *T. cruzi* gGAPDH to assess protein levels. Densitometric quantification of the signals obtained with the two antibodies allowed us to estimate the increased abundance of *Tc*ABCG1 transporter in relation to CL Brener WT; GAPDH: glyceraldehyde 3-phosphate dehydrogenase.

employed to determine differences in the protein levels among WT strains (Fig. 5B), which showed variations in *TcABCG1* transcript levels (Fig. 2). Following incubation with the anti-gGAPDH serum, densitometric quantifications of the signals indicated 2-3-fold higher abundance of the *Tc*ABCG1 transporter in strains with increased resistance to BZ, as compared to CL Brener and 115 sensitive strains (Fig. 5B). We observed a reasonable correlation between the relative abundance of *TcABCG1* transcripts and protein levels (Fig. 2).

Analysis of 5'UTR and 3'UTR of TcABCG1 genes - In kinetoplastids, regulation of gene expression is exerted at the posttranscriptional level (Clayton & Shapira 2007) and in trypanosomatids, specific motifs and ordered secondary structures in 5' and 3' UTRs may control mRNA half-life and translation efficiency (Brandão 2006, Clayton & Shapira 2007, Siegel et al. 2011, Clayton 2014).

Here we analysed *in silico* characteristics of the UTRs of *TcABCG1* genes of Silvio, Esmeraldo and CL Brener (Esmo and NEsmo), since these strains differ in BZ sensitivity (Table I), transcript abundance (Fig. 2) and protein levels (Fig. 5B).

The genomic *locus* of *TcABCG1* displays the same configuration in the three strains. It is flanked at the 5' upstream by the 40S ribosomal protein S8 (RPS8) and at 3' downstream by a hypothetical protein. The intergenic segment separating the 40S RPS8 and the *Tc*ABCG1 transporter in CL Brener NEsmo and Esmo haplotypes, Silvio and Esmeraldo strains is 526, 524, 520 and 519 bases, respectively. Intergenic mutations change both the length and nucleotide composition of the 5' UTR, which we estimated as having length of 116, 127, 121 and 123 bases, respectively, for NEsmo and Esmo haplotypes, Silvio and Esmeraldo strains. 5'UTRs were investigated with respect to the potential of generating high probability bp (through the ProbablePair algorithm). Firstly we inspected the base pairing at the highest probability threshold in each sequence. The ProbablePair algorithm allows a prediction that whatever the real conformation in that segment of the mRNA, it is almost certain that the predicted paired bases are present in the actual secondary structure. The four 5'UTR sequences analysed here exhibit paired bases at the threshold limit of 70%, that is, in the thermodynamical ensemble of secondary structures, those paired bases were present in 70% of all configurations (Fig. 6A). The low compositional variation in the 5'UTR of each strain determines the trend to be more or less structured. Apparently, the naturally BZ resistant strains (Silvio and Esmeraldo) exhibit more paired bases than both haplotypes of CL Brener BZ sensitive strain (Fig. 6A, Table IV).

The 3'UTR sequences were initially used as query to search an UTR database for similarity to conserved and functional elements from other eukaryotes. This search however, returned no elements except for the detection of two small ORFs (data not shown). Thus, the ABCG transporter 3'UTR does not possess similarity to any evolutionary conserved UTR element described so far. We next submitted the 3'UTR sequences to ProbablePair algorithm for base pairing prediction at 95% probability threshold. The predicted bp at this elevated threshold indicates that they are highly structured sequences, which is evidence that stem loop elements are almost certainly present in any secondary structure these 3'UTR might assume. A description of helix position in these sequences is available at Supplementary Table III. We then scanned the 3'UTR sequences with two RNA motif finder software (CM finder and MEME) that returned a set of three motifs each, covering more than half of 3'UTR sequence. Fig. 6B shows a diagram of detected motifs and the predicted bp for all 3'UTR segments. By this figure we see that the three algorithms produced an overlapping prediction in the segment defined by nucleotide positions 60-100 and only for two sequences (Esmeraldo and Esmo haplotype). Though the algorithm did not detect simultaneously the same element in all four sequences, the overlapping of motifs predicted by two different algorithms is a good hint that the segment spanning these motifs might be at least a hot spot for some functional activity. By this reasoning, the segment between positions 140-220 (Fig. 6B) is the one with high potential for a functional activity, because it contains several overlapping bp and has been assigned two motifs by CM finder. Analog reasoning is valid for the segment 10-100. Supplementary Table IV describes these predicted minimum common motifs. The structured nature of the 5'UTR, the highly probable stem loops and the potential sequence motifs pointed out by the *in silico* analysis of *Tc*ABCG1 3'UTRs are very suggestive that

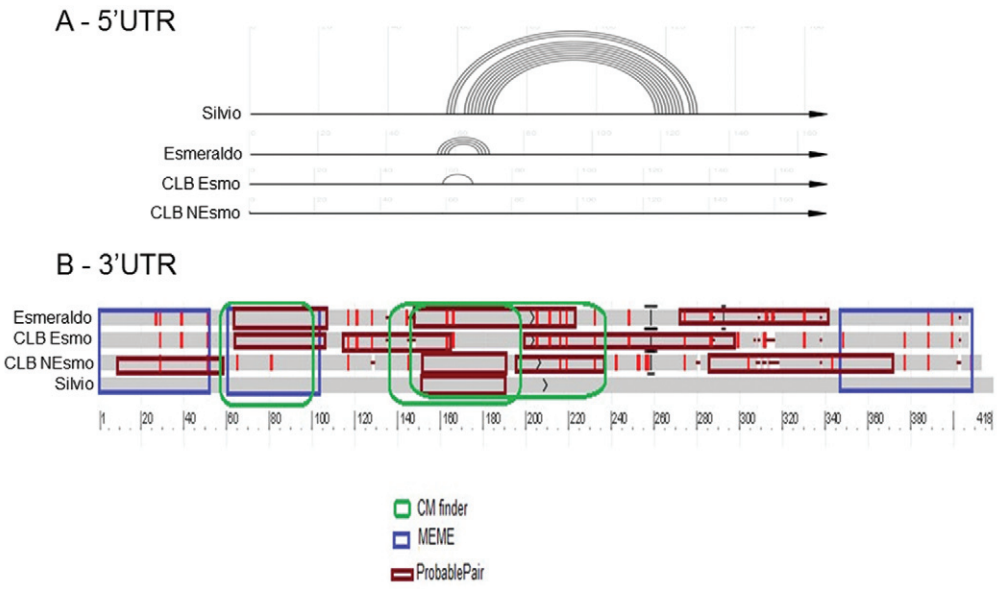

Fig. 6A: arc diagram representation of the predicted base pairs (bp) by the ProbablePair algorithm at 70% probability threshold in the estimated 5' untranslated region (UTR) sequences of *Trypanosoma cruzi* strains. A decreasing in the potential to form secondary structures in these sequences is observed from the benznidazole (BZ)-resistant strains (Silvio and Esmeraldo) to the BZ-sensitive strain (CL Brener Esmo and NEsmo); B: schematic representation of the predicted bp and potential motifs/elements detected by the algorithms ProbablePair, CM finder and MEME in the 3'UTR sequences; grey bars in the background: 400 bases of each 3'UTR; red vertical bars: nucleotide substitution sites; horizontal rectangles: predicted bp by ProbablePair at 95% threshold probability; large blue and green rectangles: position of elements identified respectively by MEME and CM finder.

TABLE IV

High probability base pairs (bp) in the 5' untranslated region (UTR) of *Tc*ABCG1 transporter[a]

Strain	bp	Helix position in 5'UTR (160 nt[b])	Stem loops	BZ phenotype
Silvio	12	57-70; 117-129	2	R
Esmeraldo	4	55-70	1	R
CL Brener Esmo	1	59-68	1	S
CL Brener NEsmo	0	-	0	S

a: paired bases appearing at the maximum threshold of 70% (ProbablePair algorithm); *b*: including spliced leader; BZ: benznidazole; R: resistant; S: susceptible.

the transcript abundance and resistance/sensitivity to BZ might be directly influenced by the variable composition of UTRs in different *T. cruzi* strains.

DISCUSSION

In this study we provide evidence that the ABCG-like transporter *Tc*ABCG1 plays a role in *T. cruzi* natural resistance to BZ. Initial indications for this were obtained from the observation that in naturally drug-resistant strains there was an increased level of *TcABCG1* gene transcripts.

We have generated CL Brener cell lines in which *TcABCG1* genes from two TcI BZ-resistant strains were independently transfected in the BZ-susceptible CL Brener strain. We verified that the transfected parasites acquired ~45% increase of BZ-resistance. Interestingly, no statistically significant increment in drug resistance was verified in CL Brener transfected with the self NEsmo haplotype. Comparison of the nucleotide sequence of *TcABCG1* gene of the TcI strains with the sequence of NEsmo haplotype showed several SNPs that produced 11 amino acid changes.

*Tc*ABCG1 transporter exhibits 29% similarity with the ABCG2 human transporter, also known as breast cancer resistance protein (Doyle et al. 1998). This transporter plays a role in multidrug resistance to chemotherapeutic agents. Amino acid changes have been identified in ABCG2, some of which affect the expression level, transporter function and/or cellular localisation (Kondo et al. 2004, Yanase et al. 2006).

We have shown that irrespective of the origin of the introduced gene, in the transfected parasites there was ~2.7-fold increased abundance of *TcABCG1* transcripts, as compared to CL Brener WT. The conserved increment of transcript abundance most likely results from the cloning of *TcABCG*1 genes in pROCK vector, in which the ORFs are flanked by the same 5' and 3' UTR sequences (DaRocha et al. 2004).

The finding that there was 2-2.6-fold increased levels of the transporter protein only in parasites transfected with TcI genes, although in agreement with the observed enhancement of drug resistance, is intriguing. Two major hypotheses could be raised to justify the results. The first one implies that characteristics of the sequence of the coding region may be influencing the translation or the steady state levels of TcI proteins. The second one argues about the expression of the protein encoded by the NEsmo haplotype. Comparison of the gene and protein sequence of Esmo and NEsmo haplotypes showed several SNPs (Supplementary Table II) and 11 amino acid changes (Table II). In addition, structural differences were verified in the 5' and 3' UTRs of the two genes (Fig. 6, Table IV). It is known that G-family transporters undergo dimerisation to perform their function (Velamakanni et al. 2007). On the other hand, it is not known if both or only one haplotype is expressed in CL Brener or if the functional *Tc*ABCG1 transporter may be formed by the dimerisation of dissimilar subunits. In the future, it will be of interest to verify if the abundance of the transporter protein increases when CL Brener is transfected with the Esmo gene.

Although CL Brener transfected with TcI genes showed ~45% increased resistance to BZ, the parasites did not attain the resistance level observed in TcI WT strains, which is 2-3-fold higher than CL Brener (Table I). This result may be due to the impaired expression of TcI genes in a TcVI strain. In this case, the dimerisation of the transporter and/or posttranslation modifications could be defective and/or additional DTU-specific elements could be required to act in conjunction with the ABCG transporter.

Our studies with WT strains suggest that the abundance of *Tc*ABCG1 transporter is a major factor for the natural resistance to BZ. In fact, we observed a reasonable correlation between the relative abundance of *TcABCG1* transcripts and protein levels.

We investigated by computational tools the characteristics of UTRs of *TcABCG1* genes of CL Brener, Silvio and Esmeraldo strains that could justify the differential gene expression in the WT strains. We estimated that 5'UTR sequences (~160 nt) of the four genes have low compositional variation and that the sequences of the naturally BZ resistant strains Silvio and Esmeraldo exhibited more paired bases than both haplotypes of CL Brener. Differences in composition and length were verified in the 3'UTR sequences (~400 nt), which could generate segments with different structural properties. For example, the resistant strain Silvio possesses a slightly longer 3'UTR and exhibits several SNPs in comparison to other strains. Structural differences were also verified between the Esmo and NEsmo haplotypes. In 3'UTRs we detected several motifs, which are overlapping segments with high probability of forming secondary structures (stem loops). This can be viewed as road map to test the hypothesis that natural acquisition of resistance to BZ might be dependent on mutational events occurring in noncoding segments like UTRs. As demonstrated by the variability in predicted bp for the sequences analysed here, a few mutations can alter the positions where a robust base pairing can appear or not in a defined segment. In the future, a more accurate analysis of the UTRs will be performed from the direct sequencing of *TcABCG1* transcripts.

For sure, even if the computational tools indicate the presence of either conserved sequence elements or high probability secondary structures in both UTRs, we cannot rule out the alternative hypothesis that the differential functional activities attributed to UTR (5' and 3') may also be the result of their interaction with trans elements, like proteins or other nucleic acids. In this direction, controlled experiments should be designed to characterise these elements.

Very few ABC genes have been characterised in *T. cruzi*, while almost 30 genes are recognised in the genome of this parasite (Leprohon et al. 2006, Sauvage et al. 2009). The first ABC genes identified in *T. cruzi* were termed *tcpgp1* and *tcpgp2* (Dallagiovanna et al. 1996, Torres et al. 1999). Because the genes belong to the ABCC family, they were renamed *TcABCC6* and *TcABCC2* (Sauvage et al. 2009). No differential expression or DNA polymorphisms of these genes between susceptible and BZ/NF-resistant *T. cruzi* strains were verified (Murta et al. 2001). One ABCA like-transporter, termed *Tc*ABCA3, seems to be involved in vesicular trafficking and is differentially expressed throughout the parasite life cycle (Torres et al. 2004). By using specific inhibitors of ABC transporters, the involvement of a P-glycoprotein in the transport of haeme through the plasma membrane of *T. cruzi* has been suggested (Lara et al. 2007).

To our knowledge, *Tc*ABCG1 is the first transporter of the G family to be characterised in *T. cruzi*. ABCG transporters are represented in *Leishmania* species. ABCG4 and ABCG6 transporters are involved in phospholipid trafficking and over-expression promoted significant resistance to miltefosine in *Leishmania infantum* (Castanys-Muñoz et al. 2007, 2008), while ABCG6 is involved in camptothecin resistance in *Leishmania donovani* (BoseDasgupta et al. 2008).

Homologous sequences of *Leishmania ABCG4* and *ABCG6* genes exist in *Trypanosoma brucei* and *T. cruzi* genomes (Leprohon et al. 2006), but their function has not been established in these organisms. The *TcABCG1* transporter here characterised has greater similarity with *Leishmania* ABCG2 transporter, implicated in phosphatidylserine translocation (Campos-Salinas et al. 2013).

Taken together, the data here presented support the proposition that *Tc*ABCG1 is involved in BZ resistance. Since members of ABCG family have been associated with cellular lipid transport, it is of interest to investigate the physiological role of *Tc*ABCG1 in *T. cruzi*. Work is in progress to assess the role of this transporter in cross-resistance to other nitro drugs. This information will be valuable in the decision of Chagas disease treatment with available drugs and drugs to be discovered in the future.

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