Experimental Parasitology 140 (2014) 33–38

Contents lists available at ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

Research Brief

Trypanosoma cruzi chemical proteomics using immobilized benznidazole

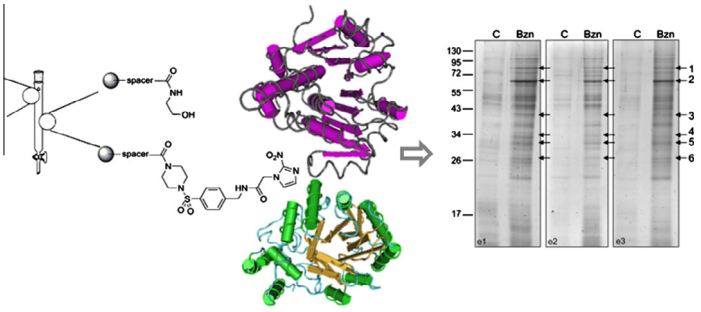
Andrea Trochine [a], Guzmán Alvarez [b], Sandra Corre [a,1], Paula Faral-Tello [a], Rosario Durán [c,d], Carlos I. Batthyany [c,d,e], Hugo Cerecetto [b], Mercedes González [b], Carlos Robello [a,e,*]

[a] Unidad de Biología Molecular, Institut Pasteur de Montevideo, Mataojo 2020, Montevideo 11400, Uruguay
[b] Grupo de Química Medicinal, Laboratorio de Química Orgánica, Facultad de Ciencias-Facultad de Química, Universidad de la República, Iguá 4225, Montevideo 11400, Uruguay
[c] Unidad de Proteómica y Bioquímica Analíticas, Institut Pasteur de Montevideo, Mataojo 2020, Montevideo 11400, Uruguay
[d] Instituto de Investigaciones Biológicas Clemente Estable, Avenida Italia 3318, CP 11600, Montevideo, Uruguay
[e] Departamento de Bioquímica, Facultad de Medicina, Universidad de la República, Montevideo, Uruguay

HIGHLIGHTS

- A Benznidazole derivative was synthesized and immobilized into a solid matrix.
- *Trypanosoma cruzi* proteins were confronted with the immobilized drug.
- Bound proteins were visualized by electrophoresis and identified by mass spectrometry.
- The aldoketo reductase TcAKR was found among the Bzn interacting partners.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 10 September 2013
Received in revised form 11 January 2014
Accepted 6 March 2014
Available online 13 March 2014

Keywords:
Benznidazole
Trypanosoma cruzi
Chemical proteomics
Aldo–ketoreductases
TcAKR

ABSTRACT

Benznidazole (Bzn) is a nitroimidazole drug currently used as first line treatment against Chagas disease, a neglected tropical disease caused by the flagellated protozoan *Trypanosoma cruzi*. Although the drug has been used since the late 1960s, its mechanism of action is not fully understood. In an attempt to study Bzn mode of action, a structurally modified derivative of the drug was synthesized and immobilized into a solid matrix. This allowed enrichment of *T. cruzi* proteins capable of binding immobilized Bzn, which were subsequently analysed by mass spectrometry. The proteins identified as specific non-covalent Bzn interactors were a homologue of the bacterial YjeF proteins, a Sec23A orthologue and the aldo–ketoreductase family member TcAKR. TcAKR is closely related to other enzymes previously associated with Bzn reductive activation such as NTRI and TcOYE. Thus, our untargeted search for Bzn binding partners allowed us to encounter proteins that could be related to drug reductive activation and/or resistance mechanisms.

1. Introduction

Chagas disease has been recognized by the World Health Organization (WHO) as one of the world's 17 most neglected tropical diseases. Although there has been a remarkable reduction in the prevalence of this disease during the last few decades, estimates indicate there are 13,000 annual deaths, 10 million people infected worldwide, and 40 million at risk of infection (Remme et al., 2006, Schofield et al., 2006). The kinetoplastid parasite *Trypanosoma cruzi* is the causative agent of this zoonosis, naturally transmitted to

Abbreviations: ROS, reactive oxygen species; MALDI-TOF/TOF MS, matrix assisted laser desorption ionization-time of flight–time of flight-mass spectrometry; NMR, nuclear magnetic resonance; TLC, thin layer chromatography; TFA, trifluoroacetic acid; MS, mass spectrometry; bs, broad singlet; d, doublet; s, singlet.

* Corresponding author at: Unidad de Biología Molecular, Institut Pasteur de Montevideo, Mataojo 2020, Montevideo 11400, Uruguay. Fax: +59 82 522 4185.
E-mail addresses: atrochine@gmail.com (A. Trochine), guzmanalvarezlqo@gmail.com (G. Alvarez), sandra.corre@pasteur.fr (S. Corre), pfaral@pasteur.edu.uy (P. Faral-Tello), duran@pasteur.edu.uy (R. Durán), batthyany@pasteur.edu.uy (C.I. Batthyany), hcerecet@fq.edu.uy (H. Cerecetto), megonzal@fq.edu.uy (M. González), robello@pasteur.edu.uy (C. Robello).
[1] Present address: Institut Pasteur, 25–28 rue du Docteur Roux, 75015 Paris, France.

http://dx.doi.org/10.1016/j.exppara.2014.03.013
0014-4894/

humans and other mammals by reduviid insects of the subfamily *Triatominae*, but also transmitted by blood transfusions, organ transplants, orally through contaminated food, and vertically from mother to child (Rassi et al., 2010).

Benznidazole (Bzn), formerly commercialized as Rochagan® and Radanil® (Roche), and Nifurtimox (Nfx), marketed as Lampit® (Bayer), are the only drugs proven effective against Chagas disease. Benznidazole has the best safety and efficacy profile, and therefore is usually used as a first line treatment. Both Bzn and Nfx have unwanted side effects that can lead to treatment discontinuation, both are genotoxic and may be carcinogenic, dosage regimes are very complex, and resistance is an increasing problem. One major limitation these drugs present is their low anti-parasitic activity in the established chronic disease, which is its most prevalent presentation (Urbina, 2010; Wilkinson et al., 2011) and the therapeutic benefits of Bzn in established mild to moderate Chagas disease are under scrutiny by the BENEFIT trial (Marin-Neto et al., 2009). Although the available drugs present certain limitations, only a few compounds are currently undergoing clinical trials against chronic Chagas disease, and there are no immediate prospects of vaccines.

Bzn and Nfx were empirically discovered as anti-trypanosomal agents without a clear understanding of their mechanisms of action. Both are nitro-heterocyclic compounds which contain a nitro group linked to an imidazole or furan ring, respectively. These agents act as pro-drugs and need to be reductively activated to have cytotoxic effects (Docampo and Stoppani, 1980; Marin-Neto et al., 2009; Wilkinson et al., 2011). Several trypanosomal enzymes have been related to the metabolism of Bzn and Nfx (Wilkinson and Kelly, 2009). Different lines of evidence suggest that a type I nitroreductase (NTR I) may act as the main reductase of both drugs *in vivo* (Wilkinson et al., 2011), generating the toxic metabolites (Hall et al., 2011; Hall and Wilkinson, 2012). Parasites overexpressing this enzyme have increased sensitivity towards the drugs, while null heterozygous mutants (Wilkinson et al., 2008), and also RNAi induced parasites targeting the NTR I transcript, display higher resistance to both Nfx and Bzn (Baker et al., 2011). Covalent binding to macromolecules including DNA and proteins has been demonstrated for Bzn (Diaz de Toranzo et al., 1988; Maya et al., 2007), as well as the decrease of low molecular weight thiols after treatment (Maya et al., 1997). Nevertheless the involvement of additional proteins on Bzn metabolism, including enzymes that may act on Bzn reduction or detoxification still remains uncertain. As a consequence, in this work we searched, by means of chemical proteomics (Menna-Barreto et al., 2014; Terstappen et al., 2007), for proteins capable of associating with an immobilized derivative of Bzn. Here we report a discrete number of Bzn binding proteins, including the aldo–ketoredutase family member TcAKR.

2. Materials and methods

2.1. Synthesis of the benznidazole derivative Bzn1 (N-(4-(piperazin-1-ylsulfonyl)benzyl)-2-(2-nitro-1H-imidazol-1-yl)acetamide)

All starting materials were commercially available research-grade chemicals and used without further purification. All solvents were dried and distilled prior to use. Melting points were determined with an electrothermal melting point apparatus (Electrothermal 9100) and were uncorrected. Proton and carbon NMR spectra were recorded on a Bruker DPX-400 spectrometer. The chemical shift values are expressed in ppm relative to tetramethylsilane, which was used as an internal standard. Mass spectra were determined using an MSD 5973 Hewlett–Packard spectrometer with electronic impact at 70 eV (EI). UV-spectra were recorded using a Varian Cary 50 Tablet UV–Visible Spectrophotometer. Column chromatography was carried out using Merck silica gel (60–230 mesh).

2.2. Synthesis of intermediate I

(Grunewald et al., 2005) Sulfurochloridic acid (15 equivalents dissolved in dichloromethane) was added dropwise to Bzn (1 equivalent) during 15 min at 0 °C and under an N_2 atmosphere. The mixture was left stirring at room temperature for 4 h. The reaction was followed by TLC (SiO_2, dichloromethane:methanol, 95:5). The product was partitioned between a phosphate buffer solution of pH 7.0 and dichloromethane (4 times) in an ice bath. It was then rapidly dried with anhydrous sodium sulphate and evaporated *in vacuo*. Finally, petroleum ether was added and evaporated *in vacuo* again, a yellow solid precipitated (intermediate (I)), yield 60%.

2.2.1. Preparation of intermediate II

N-Boc piperazine (1.05 equivalents dissolved in dichloromethane) was added dropwise, during 15 min at 0 °C and under an N_2 atmosphere, to one equivalent of intermediate (I) in presence of triethylamine (10 equivalents) and dissolved in dichloromethane (1 mL per 100 mg of intermediate (I)). After the addition was completed the mixture was left at room temperature for 12 h. The reaction was monitored by TLC (Al_2O_3, dichloromethane:methanol, 95:5). The mixture was partitioned between phosphate buffer pH 6.0–7.0 and dichloromethane. Then it was dried with anhydrous sodium sulphate and evaporated *in vacuo*. Finally petroleum ether was added and evaporated *in vacuo* again. The residue, intermediate (II) (yield 75%), was used as it in the following synthetic step.

2.2.2. Preparation of Bzn1 (N-(4-(piperazin-1-ylsulfonyl)benzyl)-2-(2-nitro-1H-imidazol-1-yl)acetamide)

Intermediate (II) was reacted with TFA (12 equivalents) dissolved in dichloromethane (3 mL). The addition was carried out dropwise over 15 min in an ice bath. After the addition was completed the mixture was left at room temperature for 24 h. At the end of the reaction, the mixture was partitioned between phosphate buffer pH 6.0–7.0 and dichloromethane, dried with anhydrous sodium sulphate and evaporated *in vacuo*. The crude was purified on a chromatographic column (Al_2O_3) with a gradient of dichloromethane: methanol (0–5%). Yellow solid, yield 20%, mp 69.0–71.0 °C. MS (EI) *m/z* (abundance,%): 409 ($M^{+\cdot}$, 100). ^{1}H NMR ($(CD_3)_2SO$) δ: 2.76 (bs, 4H), 2.78 (bs, 4H), 4.45 (d, 2H, J = 5.9 Hz), 5.21 (s, 2H), 7.21 (d, 1H, J = 1.0 Hz), 7.54 (d, 2H, J = 8.4 Hz), 7.66 (d, 1H, J = 1.0 Hz), 7.70 (d, 2H, J = 8.4 Hz), 8.32 (bs, 1H), 8.99 (bs, 1H). ^{13}C NMR ($(CD_3)_2SO$) δ: 42.0, 45.0, 47.0, 52.0, 128.0 (two carbons), 129.0 (two carbons), 134.0, 145.0 (two carbons), 167.0. λ_{max} (acetonitrile) = 320 nm (ε = 6.13 cm^{-1} mM^{-1}).

2.3. Parasites

T. cruzi epimastigotes of the DM28c strain (Contreras et al., 1988) were grown in LIT medium supplemented with yeast extract and 10% FBS at 28 °C (Camargo, 1964).

2.4. Protein extracts

Total protein extracts were prepared from exponentially growing *T. cruzi* epimastigotes (5×10^7 cells/mL). Parasites were centrifuged at 2000*g*, washed three times with cold PBS (Phosphate-Buffered Saline: 1.2 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 130 mM NaCl, 2.6 mM KCl pH 7.4) and resuspended in cold lysis buffer (150 mM NaCl, 1% Triton X-100, 0.25% NP40, 50 mM Hepes, 1 mM DTT, 1X Protease inhibitor cocktail (Sigma), 1 mM PMSF, pH 7.9). Parasites were lysed with three freeze–thaw cycles with liquid N_2 −37 °C. The lysates were cleared by centrifugation at

100,000*g* for 30 min at 4 °C. The resulting supernatants were immediately used or conserved at −80 °C until use. Proteins were quantified with Bradford reagent (Sigma).

2.5. Affinity chromatography

The benznidazole derivative, Bzn1, was covalently coupled to NHS-activated Sepharose 4 Fast Flow (GE Healthcare) as recommended by the manufacturer. NHS-activated is comprised of an N-hydroxysuccinimide (NHS) ester attached by epichloro-hydrine to Sepharose *via a* 6-atom spacer arm. Briefly, beads were washed three times with cold 1 mM HCl, immediately resuspended in coupling buffer (0.2 M $NaHCO_3$, 0.5 M NaCl pH 8) containing 10 μmol Bzn1 every 0.5 mL of packed beads, and incubated 30 min at 20 °C. After binding the free sites were inactivated with three cycles of washing with buffer A (0.5 M ethanolamine, 0.5 M NaCl pH 8) and buffer B (0.1 M sodium acetate, 0.5 M NaCl pH 4). Beads were resuspended in coupling buffer and stored at 4 °C until use. The coupling process was followed by UV-spectrophotometry. Briefly, aliquots of incubation mixture, $t = 0$ and 30 min, were measured at 320 nm. The concentration of free Bzn1 was determined from the corresponding calibration curve in acetonitrile (HPLC grade). Percentage of reaction = 58%.

For affinity chromatography, beads were washed twice with lysis buffer and incubated with protein extracts for 2 h at 4 °C. Three mg of protein were used with 25 μL of packed beads. After protein incubation beads were washed three times with wash buffer (150 mM NaCl, 0.25% NP40, 50 mM Hepes pH 7.9). Bound proteins were eluted by incubation with denaturing sample buffer (2% SDS, 10% Glycerol, 1.55% DTT, 0.002% bromophenol blue, 67.5 mM Tris–HCl) for 5 min at 95 °C. Eluted proteins were separated by SDS–PAGE in 12% acrilamide gels (Laemmli, 1970) and visualized by silver staining as previously described (Parodi-Talice et al., 2007). Attempts to elute bound proteins with up to 10 mM Bzn or Bzn1 were not successful and compound precipitation due to low water solubility hindered the use of higher drug concentrations.

2.6. Mass spectrometry analysis

Selected protein slices were treated in-gel with trypsin (Sequencing-grade Promega) overnight at 37 °C and resulting peptides were extracted using 60% acetonitrile in 0.2% TFA, concentrated by vacuum drying and desalted using C18 reverse phase micro-columns (OMIX Pippete tips, Varian). Peptide elution from the micro-columns was performed directly into the mass spectrometer sample plate with 3 μL of matrix solution (α-cyano-4-hydroxycinnamic acid in 60% acetonitrile, containing 0.2% TFA).

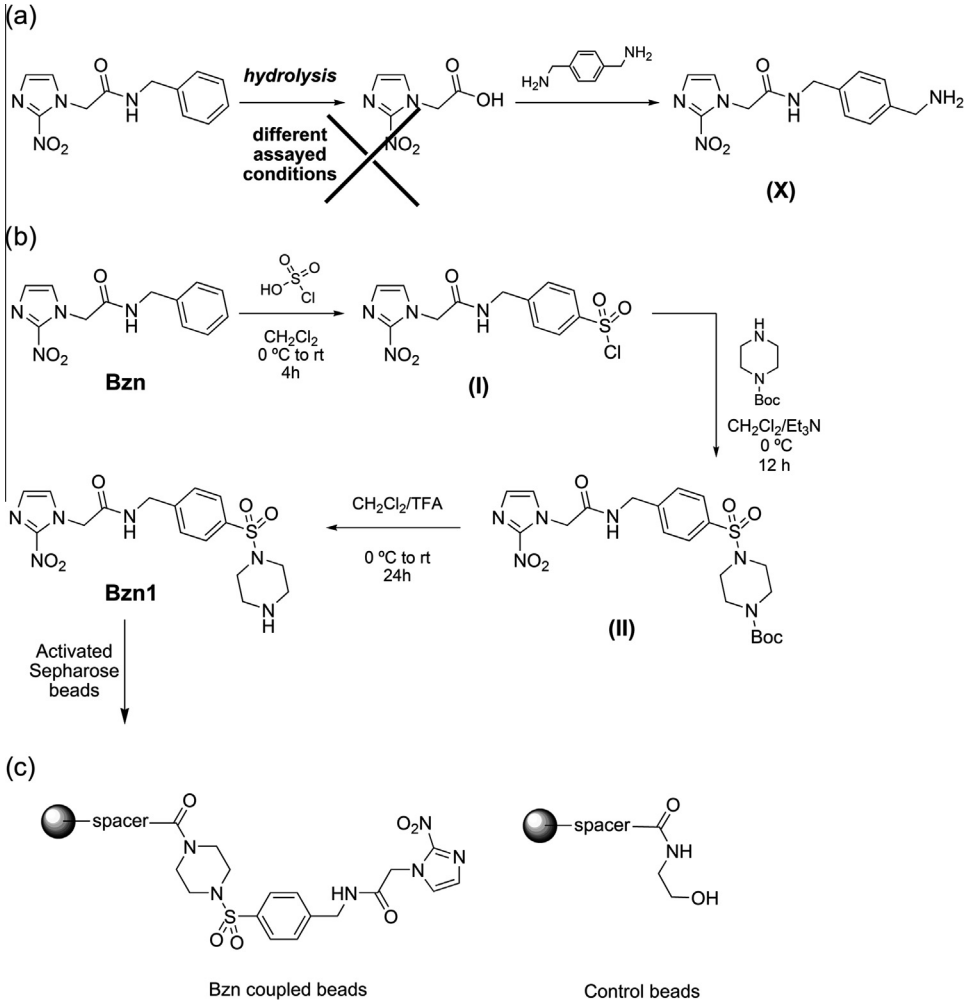


Fig. 1. Approach to obtain Bzn coupled beads. (a) Attempts to prepare Bzn derivative (X). (b) Synthetic approach to obtain nucleophilic Bzn derivative Bzn1. (c) Schematic structure of the affinity matrix obtained after reaction of Bzn1 (Bzn coupled beads) or ethanolamine (Control beads) with activated Sepharose beads. Spacer: 6 carbon spacer arm.

Mass spectra for the digestion mixtures were acquired in a 4800 MALDI-TOF/TOF instrument (Applied Biosystems) in reflector mode, externally calibrated using a mixture of peptide standards (Applied Biosystems). Collision-induced dissociation MS/MS experiments of selected peptides were performed. Proteins were identified by searching the NCBInr database (2011) using the MASCOT search engine in the "Mascot sequence query" mode (Perkins et al., 1999). The following search parameters were used: taxonomy, all entries; monoisotopic mass tolerance, 0.05 Da; fragment mass tolerance, 0.6 Da; methionine oxidation as a variable modification and one missed tryptic cleavage allowed. Significant peptide ion and protein scores ($p < 0.05$) were used as criteria for positive protein identification.

3. Results and discussion

3.1. Synthesis of a Benznidazole derivative linkable to Sepharose beads

In order to generate a Benznidazole derivative with an amino-group linkable to Sepharose beads with activated acid groups, we planned to modify the molecule in a remote position from the main pharmacophore, the 2-nitroimidazolyl moiety. This moiety has been well recognized as the responsible of the toxic effects to the parasite (Wilkinson et al., 2011) determining that other changes in the molecules could not cause any change in its mechanism of action. We designed structures that carried the nucleophilic connector, amino-group, in the benzylic position of Bzn, and two different strategies were assayed. On one hand we tried to exchange the benzylamino moiety of Bzn for a *p*-(aminomethyl)benzylamino group. The first step for this strategy was to hydrolyse the Bnz-amide moiety to later make a new amide-bond with 1,4-bis(aminomethyl)benzene, generating derivative (X) (Fig. 1a). However, we were not able to obtain the desired acid intermediate in any of the tested conditions, producing the complete decomposition of the imidazole ring. Consequently, we planned a new synthetic route (*via* intermediates (I) and (II)) as an alternative to obtain the Bzn-nucleophilic Bzn1 (Fig. 1b). After synthesis, Bzn1 was reacted into the electrophilic resin with the activated acid connector. Nearly sixty percent of the compound reacted with the activated beads as measured by UV-spectrophotometry when used in a ratio of 1:2 (compound: available bead acid sites). As control, beads were prepared under the same conditions in the absence of Bzn1 (Fig. 1c).

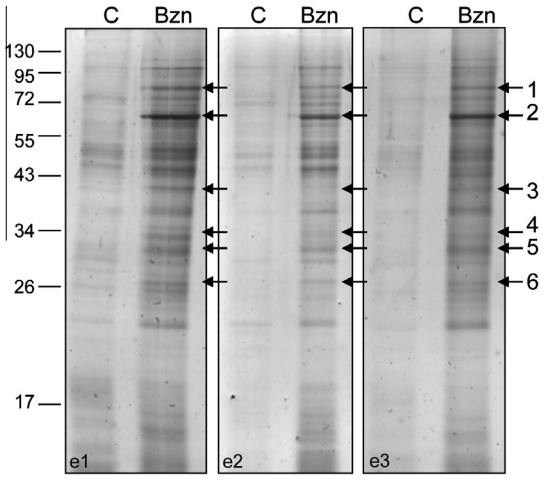


Fig. 2. Enrichment of *T. cruzi* proteins with affinity to immobilized Benznidazole. Proteins eluted from Sepharose beads with immobilized Bzn (Bzn) and from control beads (C) were separated by SDS–PAGE and visualized by silver staining. Elutions from three representative independent protein extracts are shown (e1, e2 and e3). Arrows mark bands that were cut for protein identification by MS (Table 1). Marked bands were observed in all elutions from immobilized Bzn and equivalent bands were not observed in any of the control elutions from the six independent protein extracts analyzed. Bands that did not meet these criteria were not considered for identification.

3.2. A discrete number of T. cruzi proteins bind to immobilized Benznidazole

To allow enrichment of proteins interacting with Bzn, total native *T. cruzi* protein extracts were incubated with Bzn-coupled beads. After washing, bound proteins were eluted in denaturing conditions, separated by SDS–PAGE and visualized by silver staining. Several moderate to high intensity bands were detected in the eluates from Bzn beads, whereas only very faint bands were detected in the eluates from control beads (Fig. 2). Six independent protein extracts showed the same pattern of eluted proteins. Bands detected only on Bzn eluates and absent from control eluates were excised and analysed by MALDI-TOF/TOF MS for protein identification (Table 1 and Supplementary data S1), allowing the identification of Bzn non-covalent binding partners.

Six protein bands were specifically detected in Bzn coupled beads elutions and analysed through mass spectrometry (Fig. 2 and Table 1). Several conditions were tested for enrichment of specific Bzn binding proteins. The pattern of eluted proteins was unchanged in a range of protein concentrations (20–5 μg/μL) (not shown), and was highly reproducible with independent protein extracts. Moreover, previous work on the *T. cruzi* complete proteome, using gel based methodologies (Andrade et al., 2008; Paba et al., 2004; Parodi-Talice et al., 2004), failed to detect the proteins enriched in this work after affinity with Bzn, probably due to low abundance. On the other hand, with the exception of tubulin, highly abundant proteins were not bound to immobilized Bzn, reinforcing the idea that the observed interactions are specific.

A *T. cruzi* protein of 83 kDa was identified in band 1 (Fig. 2, Table 1) showing sequence identity to the eukaryotic protein transport protein Sec23A. This gene is syntenic with the *Trypanosoma brucei* SEC23.2 gene [TriTrypDB: Tb927.10.7740], essential in the African trypanosome (Sevova and Bangs, 2009). Sec23A proteins, including *T. brucei* orthologues, are components of the Coat Protein Complex II (COP II), which coats vesicles in the endoplasmic reticulum (ER) to transport newly synthesized cargo proteins to the Golgi Complex (Barlowe et al., 1994).

The protein identified in band 2 (Fig. 2, Table 1) has a molecular weight of 63 kDa and sequence similarity to the bacterial YjeF proteins, included in the carbohydrate kinase superfamily (see Supplementary data S2). The *T. cruzi* protein has the same domain architecture as the bacterial homologues, with YjeF N and C terminal domains. The *E. coli* homologue is a non-essential protein involved in the repair of hydrated NAD(P)H. The protein is a bi-functional enzyme that dehydrates (*S*) NAD(P)HX in an ADP dependent manner and also acts as an epimerase interconverting (*R*) and (*S*) NAD(P)HX (Marbaix et al., 2011).

Tubulins were identified in three different bands (3, 4 and 6, Fig. 2 and Table 1). Even though there could be a specific interaction with Bzn, *T. cruzi* alpha and beta tubulin were identified in several bands with different apparent molecular weights using the same approach (gel based MALDI-MS proteomics) with other unrelated immobilized compounds (not shown), suggesting that they may represent a common contaminant.

A 32 kDa protein was identified in band 5 (Fig. 2 and Table 1), named TcAKR by Garavaglia and co-workers (Garavaglia et al., 2010). The protein belongs to the aldo–ketoreductase superfamily and shows a high degree of similarity (59%) with the Prostaglandin F2α synthase of *Trypanosoma brucei* (TbPGFS). TcAKR can reduce *in vitro* *o*-naphtoquinones but not *p*-naphtoquinones, although its

Table 1
Identification of Benznidazole binding proteins by MALDI-TOF/TOF MS and NCBInr database search using Mascot.

Band	Protein name	Protein ID	Theoretical MW (kDa)	Sequence coverage (%)	Protein score
1	Protein transport protein Sec23A	XP_806661	83.5	8	116 (>84)
2	YXKO-related protein	EFZ33320	63.5	20	99 (>84)
3	Alpha-tubulin	AAA99441	46.9	35	113 (>84)
4	Beta tubulin	AAA91958	49.4	36	187 (>84)
5	Prostaglandin F synthase	XP_814362	32.5	59	191 (>84)
6	Beta tubulin 1.9	AAL75956.1	49.7	44	150 (>84)

Proteins were identified using PMF data in combination with MS/MS data. Scores greater than those indicated in parentheses were considered significant ($p < 0.05$) according to the Mascot algorithm. Band numbers correspond to those shown in Fig. 2.

physiological role is still unknown and reductive activity towards Bzn has not been reported. In *T. brucei,* the overexpression of TbPGFS does not change the parasites' sensitivity towards Bzn (Hall and Wilkinson, 2012).

TcNTRI and TbNTRI diminished expression results in Nfx and Bzn resistant phenotypes (Wilkinson et al., 2011), whereas overexpression produces hypersensitivity, which stands for their critical role in the toxicity of both drugs against trypanosomes. In *T. cruzi*, the nitro-reduction of heterocyclic compounds has been related to another member of the aldo–ketoreductase family with PGF2α synthase activity: *T. cruzi* Old Yellow Enzyme (TcOYE), for which no homologue can be found in *T. brucei* genome. Recombinant TcOYE can reduce Nfx *in vitro* only under anaerobic conditions, although is not able to reduce Bzn (Kubata et al., 2002). Nevertheless, this Prostaglandin F2α synthase of 42 kDa, was found less expressed at mRNA and protein levels on Bzn *in vitro*-induced resistant parasites due to the loss of three out of four copies of the gene (Andrade et al., 2008; Murta et al., 2006). In addition, TcOYE and TcAKR have equivalent tertiary structures. TcAKR was crystallized in the apo form and its structure was recently deposited at a 2.6 Å resolution [PDB: 4fzi]. The protein has an (alpha/beta)8 barrel structure similar to other homologues including *T. brucei* TbPGFS [PDB: 1vbj], the human aldo–ketoreductases, including AKR1C3 (Komoto et al., 2004), and TcOYE (Yamaguchi et al., 2011). In these structures the cofactor $NADP^+$ and the inhibitors bind in a cavity at the C-terminal end of the barrel. With all the structural similarities TcAKR could accommodate Bzn in the same cavity.

The three *T. cruzi* enzymes, TcAKR, TcOYE and TcNTRI display quinone oxido-reductase activity (Garavaglia et al., 2010; Hall et al., 2012; Kubata et al., 2002). Remarkably, TcNTRI is proposed to act physiologically as a quinone-oxidoreductase in the mitochondrion, and possesses high catalytic specificity for synthetic quinones but lower for Nfx and Bzn, which may explain why this enzyme was not found among the immobilized proteins (Hall et al., 2012).

Different proteins may be related to Bzn metabolism in *T. cruzi* and thus related to toxicity or resistance mechanisms, including enzymes involved in drug activation, enzymes which may be related to detoxification mechanisms and proteins that may be targeted by Bzn toxic metabolites. Whether TcAKR is able to reduce or modify Bzn and which type of metabolites this reaction may render is not yet clear, but the protein drug-binding capacity and its shared features with other enzymes related to Bzn activation suggest it could have a role in Bzn mode of action in *T. cruzi*. On the other hand, the binding of *T. cruzi* Sec23A protein transport protein and of the YjeF homologue to immobilized Bzn is not easy to interpret. Some proteins may interact with immobilized Bzn trough protein–protein interactions. Additional experiments would be necessary to unveil the role of each protein, and also confirm drug–protein interactions. The use of *T. cruzi* epimastigotes allowed us to obtain high yield concentrated total protein extracts, though the use of other cultured life stages such as trypomastigotes or amastigotes would certainly represent a closer look into the putative Bzn binding partners present in the infective forms.

Several *in situ* generated Bzn metabolites, including reduction products and thiol covalent conjugates were identified in a metabolomics analysis of Bzn treated *T. cruzi* (Trochine et al., unpublished results). The multiplicity of the encountered Bzn targets and reduction products indicates that at least various enzymes may be involved in Bzn metabolism and that covalent binding to macromolecules should be further investigated. TcNTRI plays a key role in Bzn reduction to toxic metabolites but also the role of other enzymes must not be underestimated considering the genetic diversity encompassed in the six *T. cruzi* lineages so far identified, which display differences in pathogenicity, virulence, transmissibility and drug sensitivity (Andrade et al., 1985; Brisse et al., 2000).

Acknowledgments

We thank Monica Gardner (IPM) for revision of the manuscript, and Magdalena Portela (IPM) for technical assistance. This work was funded by European Union Grant agreement 223238, and research fellowships from CONICET-Argentina (A.T.) and ANII (G.A. and P.F.T.). R.D., C.I.B., H.C., M.G. and C.R. are researchers from the Sistema Nacional de Investigadores (ANII), Uruguay.

References

Andrade, H.M., Murta, S.M., Chapeaurouge, A., Perales, J., Nirde, P., Romanha, A.J., 2008. Proteomic analysis of *Trypanosoma cruzi* resistance to Benznidazole. J. Proteome Res. 7, 2357–2367.

Andrade, S.G., Magalhaes, J.B., Pontes, A.L., 1985. Evaluation of chemotherapy with benznidazole and nifurtimox in mice infected with *Trypanosoma cruzi* strains of different types. Bull. World Health Organ. 63, 721–726.

Baker, N., Alsford, S., Horn, D., 2011. Genome-wide RNAi screens in African trypanosomes identify the nifurtimox activator NTR and the eflornithine transporter AAT6. Mol. Biochem. Parasitol. 176, 55–57.

Barlowe, C., Orci, L., Yeung, T., Hosobuchi, M., Hamamoto, S., Salama, N., Rexach, M.F., Ravazzola, M., Amherdt, M., Schekman, R., 1994. COPII: a membrane coat formed by Sec proteins that drive vesicle budding from the endoplasmic reticulum. Cell 77, 895–907.

Brisse, S., Dujardin, J.C., Tibayrenc, M., 2000. Identification of six *Trypanosoma cruzi* lineages by sequence-characterised amplified region markers. Mol. Biochem. Parasitol. 111, 95–105.

Camargo, E.P., 1964. Growth and differentiation in *Trypanosoma Cruzi*. I. Origin of metacyclic trypanosomes in liquid media. Rev. Inst. Med. Trop. Sao Paulo 6, 93–100.

Contreras, V.T., Araujo-Jorge, T.C., Bonaldo, M.C., Thomaz, N., Barbosa, H.S., Meirelles Mde, N., Goldenberg, S., 1988. Biological aspects of the Dm 28c clone of Trypanosoma cruzi after metacyclogenesis in chemically defined media. Mem. Inst. Oswaldo Cruz 83, 123–133.

Diaz de Toranzo, E.G., Castro, J.A., Franke de Cazzulo, B.M., Cazzulo, J.J., 1988. Interaction of benznidazole reactive metabolites with nuclear and kinetoplastic DNA, proteins and lipids from Trypanosoma cruzi. Experientia 44, 880–881.

Docampo, R., Stoppani, A.O., 1980. Mechanism of the trypanocidal action of nifurtimox and other nitro-derivatives on *Trypanosoma cruzi*. Medicina (B Aires) 40 (Suppl. 1), 10–16.

Garavaglia, P.A., Cannata, J.J., Ruiz, A.M., Maugeri, D., Duran, R., Galleano, M., Garcia, G.A., 2010. Identification, cloning and characterization of an aldo-keto reductase from *Trypanosoma cruzi* with quinone oxido-reductase activity. Mol. Biochem. Parasitol. 173, 132–141.

Grunewald, G.L., Romero, F.A., Criscione, K.R., 2005. 3-Hydroxymethyl-7-(N-substituted aminosulfonyl)-1,2,3,4-tetrahydroisoquinoline inhibitors of phenylethanolamine N-methyltransferase that display remarkable potency and selectivity. J. Med. Chem. 48, 134–140.

Hall, B.S., Bot, C., Wilkinson, S.R., 2011. Nifurtimox activation by trypanosomal type I nitroreductases generates cytotoxic nitrile metabolites. J. Biol. Chem. 286, 13088–13095.

Hall, B.S., Meredith, E.L., Wilkinson, S.R., 2012. Targeting the substrate preference of a type I nitroreductase to develop antitrypanosomal quinone-based prodrugs. Antimicrob. Agents Chemother 56, 5821–5830.

Hall, B.S., Wilkinson, S.R., 2012. Activation of benznidazole by trypanosomal type I nitroreductases results in glyoxal formation. Antimicrob. Agents Chemother. 56, 115–123.

Komoto, J., Yamada, T., Watanabe, K., Takusagawa, F., 2004. Crystal structure of human prostaglandin F synthase (AKR1C3). Biochemistry 43, 2188–2198.

Kubata, B.K., Kabututu, Z., Nozaki, T., Munday, C.J., Fukuzumi, S., Ohkubo, K., Lazarus, M., Maruyama, T., Martin, S.K., Duszenko, M., Urade, Y., 2002. A key role for old yellow enzyme in the metabolism of drugs by *Trypanosoma cruzi*. J. Exp. Med. 196, 1241–1251.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Marbaix, A.Y., Noel, G., Detroux, A.M., Vertommen, D., Van Schaftingen, E., Linster, C.L., 2011. Extremely conserved ATP- or ADP-dependent enzymatic system for nicotinamide nucleotide repair. J. Biol. Chem. 286, 41246–41252.

Marin-Neto, J.A., Rassi Jr., A., Avezum Jr., A., Mattos, A.C., Rassi, A., Morillo, C.A., Sosa-Estani, S., Yusuf, S., 2009. The BENEFIT trial: testing the hypothesis that trypanocidal therapy is beneficial for patients with chronic Chagas heart disease. Mem. Inst. Oswaldo Cruz 104 (Suppl. 1), 319–324.

Maya, J.D., Cassels, B.K., Iturriaga-Vasquez, P., Ferreira, J., Faundez, M., Galanti, N., Ferreira, A., Morello, A., 2007. 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. 146, 601–620.

Maya, J.D., Repetto, Y., Agosin, M., Ojeda, J.M., Tellez, R., Gaule, C., Morello, A., 1997. Effects of nifurtimox and benznidazole upon glutathione and trypanothione content in epimastigote, trypomastigote and amastigote forms of *Trypanosoma cruzi*. Mol. Biochem. Parasitol. 86, 101–106.

Menna-Barreto, R.F., Belloze, K.T., Perales, J., Silva-Jr, F.P., 2014. Proteomic and Bioinformatic Analysis of *Trypanosoma Cruzi* Chemotherapy and Potential Drug Targets: New Pieces for an Old Puzzle. Curr. Drug Targets 15, 255–271.

Murta, S.M., Krieger, M.A., Montenegro, L.R., Campos, F.F., Probst, C.M., Avila, A.R., Muto, N.H., de Oliveira, R.C., Nunes, L.R., Nirde, P., Bruna-Romero, O., Goldenberg, S., Romanha, A.J., 2006. Deletion of copies of the gene encoding old yellow enzyme (TcOYE), a NAD(P)H flavin oxidoreductase, associates with in vitro-induced benznidazole resistance in *Trypanosoma cruzi*. Mol. Biochem. Parasitol. 146, 151–162.

Paba, J., Santana, J.M., Teixeira, A.R., Fontes, W., Sousa, M.V., Ricart, C.A., 2004. Proteomic analysis of the human pathogen *Trypanosoma cruzi*. Proteomics 4, 1052–1059.

Parodi-Talice, A., Duran, R., Arrambide, N., Prieto, V., Pineyro, M.D., Pritsch, O., Cayota, A., Cervenansky, C., Robello, C., 2004. Proteome analysis of the causative agent of Chagas disease: *Trypanosoma cruzi*. Int. J. Parasitol. 34, 881–886.

Parodi-Talice, A., Monteiro-Goes, V., Arrambide, N., Avila, A.R., Duran, R., Correa, A., Dallagiovanna, B., Cayota, A., Krieger, M., Goldenberg, S., Robello, C., 2007. Proteomic analysis of metacyclic trypomastigotes undergoing *Trypanosoma cruzi* metacyclogenesis. J. Mass Spectrom. 42, 1422–1432.

Perkins, D.N., Pappin, D.J., Creasy, D.M., Cottrell, J.S., 1999. Probability-based protein identification by searching sequence databases using mass spectrometry data. Electrophoresis 20, 3551–3567.

Rassi Jr., A., Rassi, A., Marin-Neto, J.A., 2010. Chagas disease. Lancet 375, 1388–1402.

Remme, J.H.F., Feenstra, P., Lever, P.R., Medici, A.C., Morel, C.M., Noma, M., Ramaiah, K.D., Richards, F., Seketeli, A., Schmunis, G., van Brakel, W.H., Vassall, A., 2006. Tropical Diseases Targeted for Elimination: Chagas Disease, Lymphatic Filariasis, Onchocerciasis, and Leprosy. In: Jamison, D.T., Breman, J.G., Measham, A.R., Alleyne, G., Claeson, M., Evans, D.B., Jha, P., Mills, A., Musgrove, P. (Eds.), Disease Control Priorities in Developing Countries, second ed. World Bank, Washington, DC, pp. 433–449 (Chapter 22).

Schofield, C.J., Jannin, J., Salvatella, R., 2006. The future of Chagas disease control. Trends Parasitol. 22, 583–588.

Sevova, E.S., Bangs, J.D., 2009. Streamlined architecture and glycosylphosphatidylinositol-dependent trafficking in the early secretory pathway of African trypanosomes. Mol. Biol. Cell 20, 4739–4750.

Terstappen, G.C., Schlupen, C., Raggiaschi, R., Gaviraghi, G., 2007. Target deconvolution strategies in drug discovery. Nat. Rev. Drug Discov. 6, 891–903.

Urbina, J.A., 2010. Specific chemotherapy of Chagas disease: relevance, current limitations and new approaches. Acta Trop. 115, 55–68.

Wilkinson, S.R., Bot, C., Kelly, J.M., Hall, B.S., 2011. Trypanocidal activity of nitroaromatic prodrugs: current treatments and future perspectives. Curr. Top. Med. Chem. 11, 2072–2084.

Wilkinson, S.R., Kelly, J.M., 2009. Trypanocidal drugs: mechanisms, resistance and new targets. Expert Rev. Mol. Med. 11, e31.

Wilkinson, S.R., Taylor, M.C., Horn, D., Kelly, J.M., Cheeseman, I., 2008. A mechanism for cross-resistance to nifurtimox and benznidazole in trypanosomes. Proc. Natl. Acad. Sci. U.S.A 105, 5022–5027.

Yamaguchi, K., Okamoto, N., Tokuoka, K., Sugiyama, S., Uchiyama, N., Matsumura, H., Inaka, K., Urade, Y., Inoue, T., 2011. Structure of the inhibitor complex of old yellow enzyme from *Trypanosoma cruzi*. J. Synchrotron Radiat. 18, 66–69.