

Putative Role of the Aldo-Keto Reductase from *Trypanosoma cruzi* in Benzimidazole Metabolism

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Benzimidazole (Bz), the drug used for treatment of Chagas’ disease (caused by the protozoan *Trypanosoma cruzi*), is activated by a parasitic NADH-dependent type I nitroreductase (NTR I). However, several studies have shown that other enzymes are involved. The aim of this study was to evaluate whether the aldo-keto reductase from *T. cruzi* (*TcAKR*), a NADPH-dependent oxido-reductase previously described by our group, uses Bz as the substrate. We demonstrated that both recombinant and native *TcAKR* enzymes reduce Bz by using NADPH, but not NADH, as a cofactor. *TcAKR*-overexpressing epimastigotes showed higher NADPH-dependent Bz reductase activity and a 50% inhibitory concentration (IC_{50}) value for Bz 1.8-fold higher than that of the controls, suggesting that *TcAKR* is involved in Bz detoxification instead of activation. To understand the role of *TcAKR* in Bz metabolism, we studied *TcAKR* expression and NADPH/NADH-dependent Bz reductase activities in two *T. cruzi* strains with differential susceptibility to Bz: CL Brener and Nicaragua. Taking into account the results obtained with *TcAKR*-overexpressing epimastigotes, we expected the more resistant strain, Nicaragua, to have higher *TcAKR* levels than CL Brener. However, the results were the opposite. CL Brener showed 2-fold higher *TcAKR* expression and 5.7-fold higher NADPH-Bz reduction than the Nicaragua strain. In addition, NADH-dependent Bz reductase activity, characteristic of NTR I, was also higher in CL Brener than in Nicaragua. We conclude that although *TcAKR* uses Bz as the substrate, *TcAKR* activity is not a determinant of Bz resistance in wild-type strains and may be overcome by other enzymes involved in Bz activation, such as NADPH- and NADH-dependent reductases.

Chagas’ disease, caused by the protozoan *Trypanosoma cruzi*, affects 11 million people in Latin America (1). The parasite is usually transmitted to humans and other mammals by triatomine bugs. However, transmission can also be oral (through contaminated food), congenital, or transfusional or by organ transplantation, even in countries where the disease is not endemic (2). The course of infection includes an acute phase followed by an indeterminate phase without symptoms, which lasts throughout life in most infected people. Approximately 30% of patients can develop a chronic phase characterized by cardiac and/or digestive pathologies that can lead to death (3).

Benzimidazole (Bz) and, to a lesser extent, nifurtimox (Nx) are the drugs currently used for treatment of *T. cruzi* infection (4). Although the mechanism of action of these nitroheterocyclic compounds is not clearly understood, it is known that, to exert their trypanocidal effects, they need to be activated through the reduction of their nitro group (5). Several studies have proposed that an NADH-dependent trypanosomal type I nitroreductase (NTR I) is a key enzyme which catalyzes Bz and Nx activation *in vivo* (6, 7, 8, 9). However, evidence indicates that although Bz is reduced to its anion radical, redox cycling may not be relevant for its mode of action, as is the case with Nx (8, 10, 11, 12, 13). Moreover, cells selected for Bz resistance display altered expression levels of several additional enzymes, indicating that other components of the parasite are involved (14, 15, 16). It has been proposed that Bz activity is mediated via highly toxic reduced intermediates that covalently bind macromolecules and cause deleterious effects, including DNA damage and thiol depletion (8, 17, 18).

We have previously described a novel NADPH-dependent aldo-keto reductase from *T. cruzi*, *TcAKR*, which reduces commonly used AKR substrates and o-naphthoquinones (o-NQ) with trypanocidal effects, such as β -lapachone (19). The generation of

free radicals is concomitant with o-NQ reduction, suggesting that *TcAKR* participates in their metabolism (19, 20, 21). Trochine et al. recently reported that *TcAKR* interacts with an immobilized derivative of Bz, suggesting that it also participates in the Bz mode of action (22). Thus, the aim of this study was to examine *TcAKR* reductase activity using Bz as a substrate and the role of this enzyme in the metabolism of this drug. Here, we showed that both recombinant and native *TcAKR* enzymes can reduce Bz using NADPH as a cofactor. Studies with transfected epimastigotes overexpressing *TcAKR* suggest that this enzyme may contribute to Bz detoxification. In addition, experiments with two *T. cruzi* strains with differential susceptibilities to Bz indicated that *TcAKR* activity may not be relevant enough to determine Bz resistance in natural strains.

MATERIALS AND METHODS

Parasite culturing and processing. The parasites used in this study were the CL Brener clone (discrete typing unit [DTU] VI), Nicaragua isolate (DTU I) (16), and Adriana (DTU I) transformed with the plasmid

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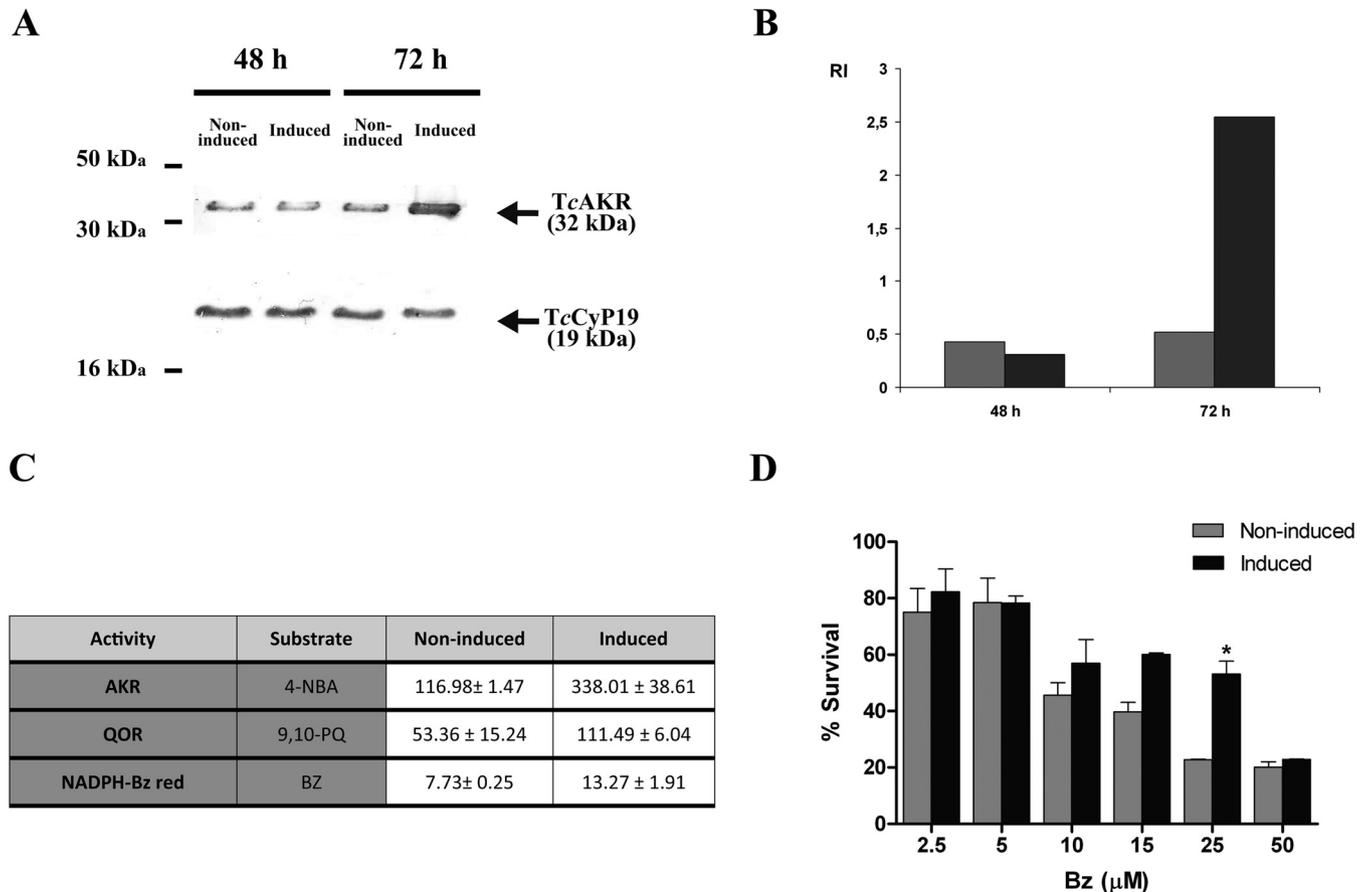


FIG 1 Overexpression of *TcAKR* and Bz susceptibility in transfected *T. cruzi* epimastigotes. (A and B) Time course of *TcAKR* expression in transfected *T. cruzi*. Noninduced and tetracycline-induced transfected epimastigotes were collected at different times and fractionated by 12% SDS-PAGE, and Western blots were probed with a mouse anti-*recTcAKR* serum. Equivalence in protein loading was controlled by immunodetection of *TcCyp19*. A representative Western blot (A) and semiquantitative analysis of *TcAKR* levels (B) are shown. Relative intensity (RI) was calculated as follows: intensity of the signal obtained with anti-*recTcAKR*/intensity of the signal obtained with anti-*TcCyp19*. (C) AKR, QOR, and NADPH-dependent Bz reductase (red) activities of *TcAKR*-transfected epimastigotes. Enzymatic activities were measured in the soluble fraction of transfected epimastigotes, either noninduced or tetracycline induced for 72 h. AKR and QOR activities were evaluated using 4-NBA and 9,10-PQ as substrates, respectively. NADPH was used as a cofactor in all the reactions. Results are expressed as nanomoles of NADPH/minute/milligram of protein. (D) Effect of Bz on the survival of *TcAKR*-overexpressing *T. cruzi*. Noninduced and tetracycline-induced pTcIndex-*TcAKR*-transfected parasites were cultured for 72 h in the presence or absence of different Bz concentrations. A survival rate of 100% corresponds to the mean number of duplicate samples of untreated epimastigotes. *, $P < 0.05$, for results in noninduced versus tetracycline-induced epimastigotes.

pLEW13 (23). Epimastigotes were cultured at 28°C in BHT medium (33 g/liter brain heart infusion medium, 3 g/liter Bacto tryptose, 0.3 g/liter glucose, 5.4 mM KCl, 28.2 mM Na_2HPO_4 , 0.002% [wt/vol] hemin) supplemented with 10% (vol/vol) of fetal bovine serum (FBS). Transformed pLEW13 Adriana stock was cultured with 200 $\mu\text{g}/\text{ml}$ G418 and hygromycin.

To obtain the soluble fraction of parasites, epimastigotes were suspended in 100 mM Tris-HCl, pH 7.4 (2×10^9 parasites in 1 ml of buffer), and ruptured by freeze-thawing with liquid N_2 ; afterwards, lysates were centrifuged at $10,000 \times g$ for 30 min at 4°C. The supernatant (soluble fraction) was used to measure enzymatic activities or purify native *TcAKR*.

Recombinant and native *TcAKR* purification. Expression and purification of recombinant *TcAKR* (*recTcAKR*) were performed as previously described from *Escherichia coli* (M15) transformed with the plasmid pQE30-*TcAKR* (19). Native *TcAKR* was purified from the soluble fraction of CL Brener clone epimastigotes by Cibacron Blue-Sepharose CL-6B resin (Pharmacia Biotech) eluted with 0.7 mM NADPH (19).

Enzymatic assays. Reducing activities were routinely assayed by measuring NADPH or NADH oxidation at 340 nm ($\epsilon = 6,270 \text{ M}^{-1} \text{ cm}^{-1}$) at 30°C in a Beckman Coulter DU-640UV instrument.

AKR activity was determined in reaction mixtures of 0.25 ml containing 5 to 150 μg of the soluble fraction from epimastigote lysates in 100 mM Tris-HCl buffer, pH 6.5, with 1 mM 4-nitrobenzaldehyde (4-NBA) and 0.2 mM NADPH.

Quinoneoxido-reductase (QOR) activity was evaluated using 0.025 mM 9,10-phenanthrenequinone (9,10-PQ) in a final volume of 0.1 ml containing 100 mM triethanolamine buffer, pH 7.7, 0.1 mM NADPH, 0.002 mM EDTA, and 25 to 120 μg of the soluble fraction.

Bz reductase activity was measured in the same reaction mixture used for AKR activity but with 4-NBA replaced by 0.1 mM Bz. In this case, 20 μg and 5 to 20 μg of recombinant and native *TcAKR*, respectively, or 25 to 250 μg of the soluble fraction was used. To assess NADH-dependent Bz reductase activity, NADPH was replaced by 0.1 mM NADH.

***TcAKR* subcloning, parasite transfection, and *TcAKR* overexpression.** The *TcAKR* gene was excised from the recombinant plasmid pGEM-*TcAKR* (19) with NotI and introduced into the inducible expression vector pTcIndex (23) excised with the same restriction enzyme to form pTcIndex-*TcAKR*. This plasmid was introduced by electroporation into Adriana epimastigotes previously transformed with plasmid pLEW13. For electroporation, parasites in the mid-log phase were washed and suspended in BHT medium at a final concentration of 5×10^8 cells per ml.

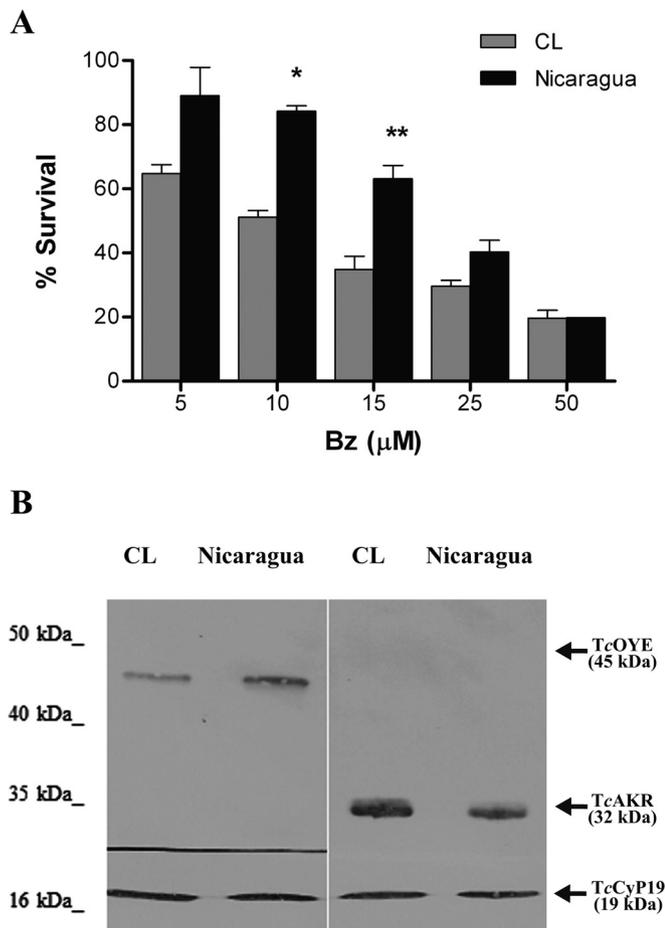


FIG 2 Bz susceptibility and *TcAKR* expression in *T. cruzi* CL Brener and Nicaragua. (A) Effect of Bz on the survival of epimastigotes of *T. cruzi* CL Brener and Nicaragua. Parasite growth was monitored after 72 h of culture in the presence or absence of different Bz concentrations. A survival rate of 100% corresponds to the mean number of duplicate samples of untreated epimastigotes. *, $P < 0.05$; **, $P < 0.01$, for results with CL Brener versus those with Nicaragua. (B) Analysis of *TcAKR* protein expression by Western blotting in *T. cruzi* CL Brener and Nicaragua. Western blot analysis of proteins derived from epimastigotes of CL Brener and Nicaragua *T. cruzi* strains was performed, as indicated, with mouse anti-*recTcAKR* polyclonal serum (right panel), rabbit anti-*TcOYE* N-terminal peptide polyclonal serum (left panel), and rabbit anti-*recTcCyp19* polyclonal serum (both panels). *TcCyp19* protein was used as a loading control.

Aliquots of 0.35 ml were dispensed into disposable 0.4-mm cuvettes containing 10 μg of plasmid DNA, and cells were electroporated by using a Bio-Rad gene pulser at 335 V and 1,400 mF, with two consecutive pulses. After 5 min on ice, cells were diluted 10-fold with BHT medium containing 10% tetracycline-free FBS (Natocor) and allowed to recover for 24 h. G418 and hygromycin B (200 μg/ml) were added, and parasites were incubated at 28°C. Transgenic parasites were obtained after 3 weeks of selection with both antibiotics. Epimastigote cultures were grown to reach a cell density of 5×10^6 parasites per ml, and protein expression was induced by the addition of 5 μg/ml tetracycline.

Adriana epimastigotes transfected with the green fluorescent protein (GFP) gene were used as controls in all the assays. GFP expression by tetracycline induction was evaluated by optical microscopy in a Leica DM 2500 fluorescence microscope.

Growth inhibition assays and IC₅₀ calculation. To determine the sensitivity to Bz, epimastigotes (5×10^6 cells/ml) were seeded into BHT

medium–10% fetal calf serum (FCS) in 96-well culture flasks (200 μl/well) in the presence of increasing amounts of Bz (0 to 50 μM) solubilized in dimethyl formamide (DMFA) and cultured for 72 h. Transfected parasites were grown with 200 μg/ml G418 and hygromycin, and, to induce the overexpression of these proteins, 5 μg/ml of tetracycline was added 1 day before the starting of the assay. The final concentration of DMFA was fixed below the toxic level (1%). Parasite number was determined by optical microscopy counting in a Neubauer chamber. Untreated controls reached a cell density of about 33×10^6 parasites/ml after 72 h of culture, whereas untreated tetracycline-induced *TcAKR*-transfected parasites reached 18×10^6 parasites/ml. Parasite growth in the absence of Bz was considered 100%, and the concentration of drug that produced death in 50% of parasites (IC₅₀) was calculated by a dose-response curve using nonlinear regression analysis carried out with Prism, version 5.0, software (GraphPad, San Diego, CA).

Intracellular ROS and ΔΨ_m detection. Epimastigotes were grown as described in growth inhibition assays with Bz concentrations based on the IC₅₀s. After 72 h of treatment, epimastigotes were washed, and pellets were resuspended in BHT medium. To evaluate intracellular reactive oxygen species (ROS), cells were incubated with 10 μM dichlorodihydrofluorescein diacetate (H₂DCF-DA), and to evaluate the mitochondrial membrane potential (ΔΨ_m), cells were incubated with 10 μg/ml rhodamine 123 for 30 min at 28°C. Hydrogen peroxide (0.5 mM) and 20 μM carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) were used as positive controls for ROS production and ΔΨ_m detection, respectively. After incubation with the probes, cells were collected and suspended in phosphate-buffered saline (PBS). Fluorescence was detected in a flow cytometer (FACSCalibur; Becton Dickinson and Co., NJ, USA), and Cyflogic software, version 1.2.1, was used for the data analyses. A total of 10,000 events were acquired in the region previously established as the one that corresponded to the parasites.

Western blot analysis of *TcAKR* expression. Epimastigote pellets (2.5×10^5 parasites/lane) were separated by 12% SDS-PAGE and electroblotted onto nitrocellulose membranes. Blots were blocked with 10% (vol/vol) skim milk in PBS and incubated for 1 h at room temperature with mouse anti-*recTcAKR* serum (1:500). The membrane was washed with PBS and then incubated for 1 h at room temperature with biotinylated anti-mouse IgG (Vector) diluted 1:2,000. After washes, membranes were incubated with streptavidin-horseradish peroxidase (HRP) (Vector) diluted 1:1,000 for 30 min at room temperature (RT). To quantify *TcAKR* expression, *TcCyp19* was used as a loading control and detected with rabbit anti-*recTcCyp19* serum (1:1,000). Rabbit anti-*T. cruzi* old yellow enzyme (*TcOYE*) N-terminal peptide serum (1:500) was used to detect *TcOYE* expression. In both cases, after primary antibody treatment, membranes were incubated with mouse anti-rabbit IgG-HRP (Jackson) (1/2,000). Detection was performed with 4-chloro-1-naphthol, and bands were scanned and quantified using ImageJ software (version 1.410).

Statistical analysis. Each experiment was performed in duplicate in at least three independent assays. Parameters are expressed as the mean values ± standard error of the mean (mean ± SEM). Results were analyzed by a Mann-Whitney test. Differences were considered to be statistically significant at a P value of < 0.05 .

RESULTS

Reductase activity of *TcAKR* using Bz as the substrate. A recent publication reporting that *TcAKR* interacts with an immobilized derivative of Bz (22) led us to test the hypothesis that this enzyme may use Bz as a substrate. Both *recTcAKR* and the native enzyme purified from epimastigotes by Cibacron Blue affinity chromatography eluted with NADPH (19) showed reductase activity toward Bz using NADPH, but not NADH, as a cofactor, with specific activities of 90.58 ± 6.96 and 73.11 ± 3.65 nmol of NADPH/min/mg of protein, respectively. It is worth mentioning that native *TcAKR* did not coelute with trypanothione reductase (TR) or *T. cruzi* old yellow enzyme (*TcOYE*) since Western blot assays using

TABLE 1 Relation between TcAKR activity and Bz susceptibility in epimastigotes of CL Brener and Nicaragua strains of *T. cruzi*

Parameter	Value for the parameter by strain	
	CL Brener (DTU VI)	Nicaragua (DTU I)
TcAKR expression (<i>n</i> -fold) ^{a,i}	3.45 ± 0.46	1.86 ± 0.51
NADPH-dependent enzymatic activity (nmol NADPH/min/mg)		
AKR ^{b,i}	365.37 ± 25.64	137.31 ± 8.72
QOR ^c	140.92 ± 13.84	44.58 ± 7.53
Bz reductase ^{d,i}	16.01 ± 0.65	2.80 ± 0.73
NADH-dependent enzymatic activity (nmol NADH/min/mg)		
Bz reductase ^{e,i}	19.43 ± 1.44	8.24 ± 1.31
Bz trypanocidal activity		
IC ₅₀ (μM) ^{f,i}	10 ± 0.095	20.50 ± 0.30
ROS (RFU) ^g	4.58 ± 0.88	3.63 ± 0.22
ψm ^h	0.28 ± 0.02	0.22 ± 0.02

^a Ratio of the densitometric values of TcAKR and TcCyp19 bands obtained in Western blot assays as shown in Fig. 2B.

^b AKR activity using 4-NBA as a substrate.

^c QOR activity using 9,10-PQ as a substrate.

^d Bz reduction using NADPH as a cofactor.

^e Bz reduction using NADH as a cofactor.

^f IC₅₀s calculated by linear regression analysis of the plot of the growth constant versus drug concentration (Fig. 2A).

^g Detection of intracellular ROS production by flow cytometry using the H₂DCF-DA probe of Bz-treated epimastigotes. Values correspond to the relative fluorescence units (RFU) calculated as the median fluorescence intensity (MFI) of Bz-treated parasites/MFI of untreated parasites.

^h Measurement of change in mitochondrial membrane potential (Δψm) by flow cytometry using rhodamine 123 of Bz-treated epimastigotes. Values correspond to the relative fluorescence units (RFU) calculated as the median fluorescence intensity (MFI) of Bz-treated parasites/MFI of untreated parasites.

ⁱ *P* < 0.05%, for the difference between results with CL Brener and Nicaragua.

specific sera against both enzymes did not react with the NADPH-eluted fraction (data not shown). When Nx was tested as the substrate of recTcAKR, no activity was detected with either NADH or NADPH as a cofactor.

Susceptibility to Bz of transfected epimastigotes overexpressing TcAKR. To understand the role of TcAKR in Bz metabolism, we genetically engineered epimastigotes for tetracycline-inducible overexpression of TcAKR. Time course evaluation of TcAKR expression by transfected parasites was performed by Western blotting using mouse anti-recTcAKR serum. Tetracycline-induced parasites showed about a 2.5-fold increase in TcAKR expression after 72 h of induction (Fig. 1A and B). Accordingly, AKR and QOR activities were increased to the same extents. NADPH-dependent Bz reductase activity was also increased after induction, confirming that TcAKR uses Bz as the substrate (Fig. 1C).

When sensitivity to Bz of TcAKR-transfected epimastigotes was evaluated, TcAKR-overexpressing cells were more resistant to Bz, showing an IC₅₀ value 1.8-fold higher than the values of the noninduced controls (Fig. 1D). TcAKR-transfected parasites shifted the IC₅₀ from 9.90 ± 1.30 μM to 17.45 ± 1.25 μM after tetracycline induction. This suggests that TcAKR may not be involved in Bz activation but may participate in Bz detoxification.

TcAKR expression in two *T. cruzi* strains with differential susceptibilities to Bz. To evaluate the significance of TcAKR activity in Bz metabolism, we studied TcAKR expression and its related enzymatic activities in two *T. cruzi* strains with differential susceptibilities to Bz: CL Brener and Nicaragua (24). CL Brener was more susceptible to Bz than Nicaragua, presenting IC₅₀s of 10 and 20.5 μM, respectively (Fig. 2A and Table 1), and showed higher TcAKR levels (Fig. 2B and Table 1), as evaluated by Western blotting using anti-recTcAKR serum. As a reference, the ex-

pression of TcOYE, an NAD(P)H oxidoreductase which does not reduce Bz (25), was also tested and found to be slightly higher in Nicaragua than in CL Brener (Fig. 2B). In correlation with TcAKR expression, CL Brener presented higher AKR, QOR, and NADPH-dependent Bz reductase activities than Nicaragua (Table 1). However, while TcAKR expression and AKR and QOR activities were about 2.8-fold higher in CL Brener than in the Nicaragua strain, NADPH-dependent Bz reductase activity was increased 5.7-fold, suggesting that enzymes other than TcAKR may reduce Bz using NADPH as a cofactor.

Based on the results of TcAKR-overexpressing epimastigotes, we expected TcAKR expression and activity to be lower in CL Brener (more susceptible) than in Nicaragua (more resistant). Therefore, we speculated that TcAKR activity might be overcome by other enzymes of the parasite involved in the activation of Bz, such as NADPH- or NADH-dependent reductases. In this context, we measured the NADH-dependent Bz reductase activity as an indication of the Bz-activating enzyme NTR I and found that it was higher in CL Brener than in Nicaragua (Table 1), indicating an association between the characteristic NTR I activity and Bz susceptibility.

As the participation of ROS in the Bz mechanism of action is still controversial, ROS production and Δψm were evaluated by flow cytometry using specific probes in Bz-treated parasites of both lineages. Treatment with Bz induced significant ROS production and ψm decreases in both CL Brener and Nicaragua compared to levels in untreated controls (Fig. 3). However, no differences between the two lineages were found when relative levels of ROS production and ψm decrease were analyzed (Table 1), suggesting that Bz reduction by NADH and NADPH-dependent reductases may not generate ROS and, as stated by others (8, 10),

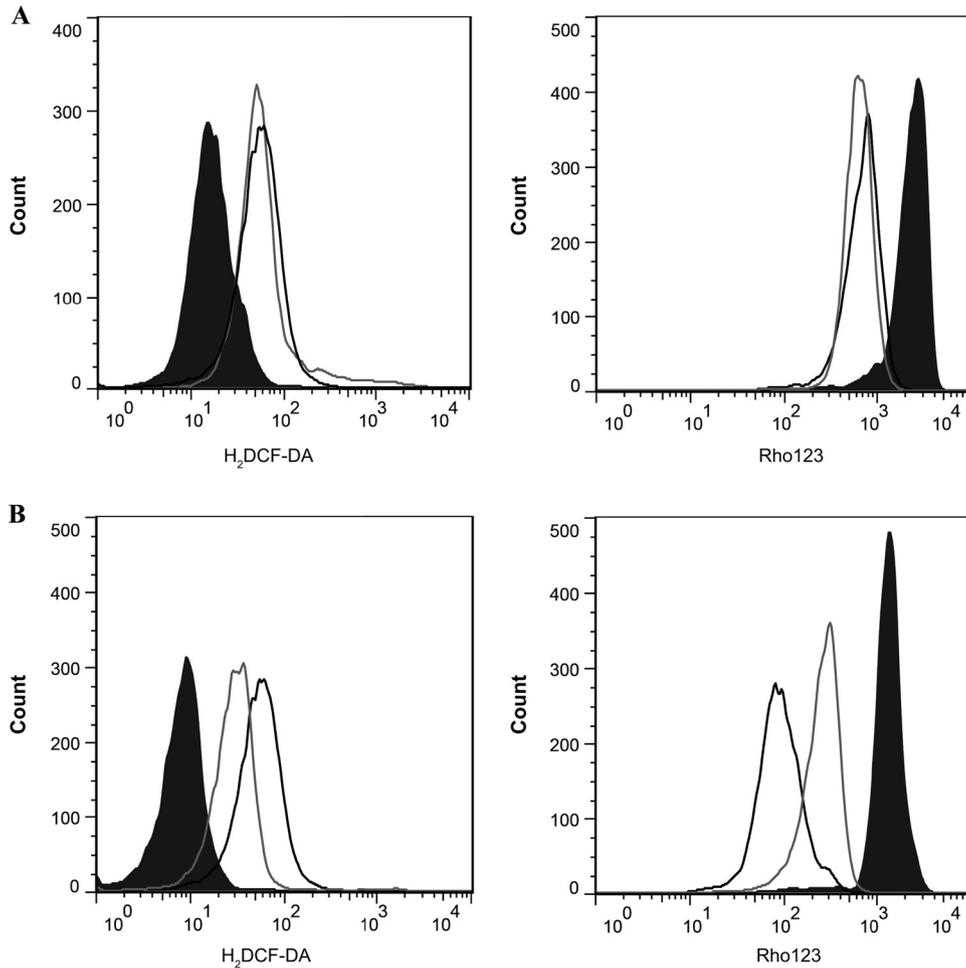


FIG 3 ROS production and mitochondrial membrane potential of CL Brener and Nicaragua epimastigotes treated with Bz. Shown are representative histograms corresponding to fluorescence analysis of epimastigotes of CL Brener (A) and Nicaragua (B) strains, either untreated or treated with 50 μM Bz and stained with H₂DCF-DA (ROS) or rhodamine 123 ($\Delta\Psi\text{m}$). The black histograms represent untreated parasites. The black line corresponds to a positive control performed with either 0.5 mM H₂O₂ for ROS production or 20 μM carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) for $\Delta\Psi\text{m}$ detection. The gray line corresponds to parasites treated with 50 μM Bz.

that another mechanism different from ROS production may be responsible for Bz cytotoxicity.

DISCUSSION

Here, we demonstrated that not only recombinant but also native *TcAKR* enzymes of *T. cruzi* reduce Bz using NADPH as a cofactor, with specific activity ranges in the same order of magnitude. These findings were supported by *TcAKR*-overexpressing parasites, which showed increased NADPH-dependent Bz reductase activity and higher resistance to this drug, suggesting that this enzyme may be involved in Bz detoxification. In addition, the NADPH-dependent reduction of Bz by *TcAKR* is consistent with the affinity of the natural enzyme to a Bz derivative coupled to a solid matrix found by Trochine et al. (22). It is important to note that the catalytic activities toward Bz are very difficult to measure because they are very poor and because Bz and the cofactor react nonenzymatically. Nevertheless, the Bz reductase activity of *TcAKR* was only slightly lower than that of recombinant NTR I (7, 26). However, unlike NTR I, a 2- to 4-fold increase in *TcAKR* expression modified the Bz resistance phenotype of transgenic parasites only mildly.

Throughout the work, *TcAKR* levels evaluated by Western blotting correlated with AKR, QOR, and NADPH-dependent Bz reductase activities. To our knowledge, besides *TcAKR*, no other enzymes of the AKR superfamily have been described in *T. cruzi*, and although several NADPH-dependent oxido-reductases have been identified (25, 27), we have shown that *TcAKR* has the highest abundance (19). Hence, we may assume that AKR activity, measured using 4-NBA as the substrate, reflects the amount of *TcAKR* present in the parasite lysate. In contrast, as other *T. cruzi* oxido-reductases display QOR activity (25, 26), this cannot be taken as a good indicator of *TcAKR* levels. The results reported here also indicate that we cannot ascribe NADPH-dependent Bz reductase activity solely to *TcAKR*.

To understand the importance of the NADPH-dependent reduction of Bz by *TcAKR* in nature, we studied this enzyme in two Bz-susceptible *T. cruzi* strains with significantly different IC₅₀s. Taking into account the results obtained with *TcAKR*-overexpressing epimastigotes, we expected the more resistant strain, Nicaragua, to have higher *TcAKR* levels than CL Brener. However, the results were the opposite. As the only parasite enzyme that has

been demonstrated to play a key role in Bz activation is NTR I, which uses NADH as a cofactor (7, 8), we evaluated the NADH-dependent Bz reductase activity in both strains. Since NTR I is a mitochondrial enzyme, before measuring its activity, we ensured the release of matrix material from mitochondria by checking the presence of cytochrome *c* in the soluble fraction by Western blotting (data not shown). In line with previous publications (8), the NADH-dependent Bz reductase activity was higher in CL Brener than in Nicaragua. In addition, that CL Brener showed higher NADPH-dependent Bz reductase activity than Nicaragua and that this activity was higher than that attributable to TcAKR suggest that Bz may be also activated through NADPH-dependent reductases.

The participation of ROS in the Bz mechanism of action is still controversial. It has been documented that Bz is reduced to its anion radical. However, the level of ROS detected seems to be insufficiently high to inhibit *T. cruzi* growth (5, 10, 12). To better understand the mechanism of action of Bz, we evaluated ROS production and $\Delta\Psi_m$ in Bz-treated epimastigotes using a technique different from that used in previous studies: flow cytometry with the specific probes, H₂DCF-DA and rhodamine 123, respectively. In order to determine whether there is a correlation between changes in these two parameters and susceptibility to Bz, the experiments were performed in two *T. cruzi* strains with not only differential susceptibilities to Bz but also different TcAKR expression levels and NADH and NADPH-dependent Bz reductase activities. We demonstrated that treatment with Bz produces ROS and $\Delta\Psi_m$. However, since these effects were observed to the same extent in CL Brener and Nicaragua, we may infer (i) that the IC₅₀ differences between the two strains are not due to these alterations and (ii) that Bz reduction by NADH and NADPH-dependent reductases may not generate ROS. This result is in agreement with previous reports demonstrating that the trypanocidal effect of Bz is exerted by reduced metabolites of the drug interacting with macromolecules from the parasite rather than by ROS formation and $\Delta\Psi_m$ (17, 18, 28).

Approaches to understand the Bz mechanism of action have yielded a wide range of proteins (14, 15, 16, 22, 29), but regulation of neither NTR I nor TcAKR has been detected. There is no documentation regarding Bz detoxification pathways, and no enzymes have so far been demonstrated to be involved in this mechanism. However, our results suggest that TcAKR is one of the enzymes participating in this process. Future research about the catalytic mechanism used by TcAKR with Bz as the substrate may elucidate this unknown metabolism. Our studies with TcAKR-overexpressing epimastigotes and *T. cruzi* strains with differential susceptibilities to Bz allow the conclusion that although TcAKR uses Bz as a substrate, the Bz reductase activity of TcAKR is not a determinant of Bz resistance.

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