Interaction of benznidazole reactive metabolites with nuclear and kinetoplastic DNA, proteins and lipids from *Trypanosoma cruzi*

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Summary. Epimastigotes of *Trypanosoma cruzi* (Tulahuen strain Tul 0 stock) biotransform benznidazole (N-benzyl-2-nitro-1-imidazole acetamide) to reactive metabolites that bind covalently to DNA, proteins and lipids of the parasite. These effects might be related to the trypanocidal action of benznidazole, a chemotherapeutic agent against Chagas' disease.

Key words. Trypanosoma cruzi; benznidazole; Chagas' disease; American trypanosomiasis; chemotherapy.

Two drugs are currently in use as chemotherapy for the acute phase of Chagas' disease; Nifurtimox (Nfx), which is a nitrofurane, and benznidazole (Bz), which is a nitroimidazole[1,2]. Generation of Nfx nitroanion radical with concomitant production of $O_2^{\cdot-}$ and H_2O_2 by autooxidation has been related to the trypanocidal action of the drugs and has been suggested as a potential mechanism for their toxic effects on mammals[1-6]. However, the mechanism of action of Bz is different from that of Nfx both in *T. cruzi* and in mammals[1-6]. For example, Bz, unlike Nfx, inhibits *T. cruzi* respiration rather than stimulating it[1,3-6], and does not lead to $O_2^{\cdot-}$ and H_2O_2 production at concentrations able to inhibit *T. cruzi* growth[1,3-6]. Bz nitroanion radical is only observed when Bz is added to *T. cruzi* homogenates in relatively high concentrations[1,3-6]. In experiments with mammals, Bz is not able to stimulate lipid peroxidation in vivo[7]. Notwithstanding, Bz reactive metabolites, generated during the nitroreduction by mammalian liver NADPH cytochrome P-450 reductase, P-450, xanthine oxidase and aldehyde oxidase, bind to DNA, proteins and lipids[8-11]; this reaction has been suggested as a potential mechanism for the toxic effects of Bz observed in mammals.

In this work we report that epimastigotes of *T. cruzi* bioactivate Bz to form reactive metabolites that bind covalently to nuclear (n) and kinetoplast (k) DNA, proteins and lipids of the parasite.

Materials and methods. Chemicals: Benznidazole (Bz) (N-benzyl-2-nitro-1-imidazole acetamide) and 2- ^{14}C-Bz (sp. act. 58 μCi/mg) were gifts from Hoffmann-La Roche & Co. (Switzerland).

T. cruzi preparations: Epimastigotes of *T. cruzi* (Tulahuen strain Tul 0 stock) were cultured and harvested as previously described[12]. The organisms were grown at 28 °C in a liquid medium made of brain-heart infusion (Difco), 37 g; hemin, 20 mg; fetal bovine serum, 40 ml; and water to 1 l. The cells, in the exponential phase of growth, were collected by centrifugation, washed twice and resuspended in 0.14 M NaCl, 3 mM KCl, sodium phosphate buffer pH 7.4 (PBS).

Procedures: T. cruzi epimastigotes (4×10^8 to 7×10^8 cells/ml) in a final volume of 2.5 ml were incubated at 28 °C in PBS buffer pH 7.4 with ^{14}C (Bz) (final concentration 0.28 mM for proteins and lipids and 0.15 mM for DNA). Reactions were stopped by addition of 15% trichloroacetic acid (TCA) for isolation of proteins as previously described[14].

Proteins were then precipitated with TCA, washed three times with 5% TCA and heated for 30 min between 85 and 90 °C to eliminate nucleic acids. The TCA precipitate was further washed successively with ethanol-diethyl ether-chloroform (2:2:1, v/v) until radioactivity in the organic phase reached the background level, then with acetone and diethyl ether for removing lipid, and finally dried.

Radioactivity was determined by dissolving the samples in formic acid and counting in a liquid scintillator. 15% TCA was added before the 3-h incubation to the sample corresponding to 0-h incubation time.

For isolation of lipids, reactions were stopped by heating at 100 °C for 10 min. The zero sample was heated before the 3-h incubation. Separation of lipids into different classes was performed as previously described[16]. Total lipids were separated into different classes by chromatography on silica gel G using petroleum ether-ethyl ether-acetic acid (80:20:1, v/v) as developing solvent. Phospholipids and Bz, which remained at the origin, were eluted and chromatographed again on silica gel G. The separation of phospholipids, which remained at the starting point in this chromatogram, from unreacted Bz, Rf 0.44, was attained by using chlorform-acetone-acetic acid (85:10:7.5, v/v) as developing solvent. Each lipid fraction was extracted, dried and counted in a liquid scintillator. Pure standards were run simultaneously.

For isolation of DNA, reactions were stopped by addition of 10% sodium dodecyl sulfate (SDS). Total DNA was extracted as described by Borst et al.[13] with some modifications. 10% SDS was added before the 3-h incubation to the zero sample. Lysed cells were incubated overnight at room temperature in a solution containing 1% SDS (final concentration); 12 ml of buffer 100 mM NaCl, 100 mM sodium EDTA, 10 mM Tris-HCl, pH 7.5 and pronase 1 mg/ml (final concentration). The incubation mixture was extracted with an equal volume of chloroform/isoamylic alcohol/phenol (48:2:50, v/v) (CIP) and centrifuged for 15 min at 17,000 g twice. n-DNA was separated from k-KNA by centrifuging the aqueous upper layer containing total DNA at 16,000 g for 30 min. The k-DNA precipitate was washed with buffer, centrifuged at 16,000 g and incubated at room temperature for 12 h, with previous resuspension in a solution containing 5 ml buffer 10 mM Tris-HCl, 1 mM EDTA, pH 7.5; 0.1% SDS (final concentration) and 0.5 mg/ml pronase (final concentration). n-DNA was precipitated from the supernatant by adding 2 volumes of ethanol and keeping the sample at −20 °C for at least 2 h. The n-DNA was then incubated at room temperature; first for 7 h, with previous resuspension, in 10 ml buffer 10 mM Tris-HCl, 1 mM EDTA, pH 7.5 and 100 μg RNase/ml (final concentration), and then for 12 h with prior addition of SDS (0.1% final concentration) and pronase (0.5 mg/ml final concentration). After incubation, both n- and k-DNA were deproteinized by adding an equal volume of CIP to each incubation mixture, precipitated with 2 volumes of ethanol, and stored at −20 °C. Once they had been centrifuged and the supernatants decanted, both DNAs were dried in vacuo for 2–3 h. The amount of DNA was determined on the basis of an absorbance of 20 at 260 nm for a solution of 1 mg DNA/ml. Radioactivity was determined by dissolving the samples and counting in a liquid scintillator. Blank values corresponding to zero incubation time were substracted from experimental values at 0.5, 1 and 3 h to correct for non-specific binding of ^{14}C(Bz).

Covalent binding (CB) of benznidazole (Bz) reactive metabolites to proteins, lipids and n- and k-DNA from *T. cruzi*

Incubation time (h)	CB of Bz (pmol Bz bound/mg) Proteins	Lipids	n-DNA	k-DNA
0	0	0	0	0
0.5	566	4110	419	0
1	935	7814	558	0
3	1833	12,147	1074	4318

Trypanosoma cruzi epimastigotes (4×10^8 to 7×10^8 cells/ml) in a final volume of 2.5 ml were incubated at 28 °C in PBS buffer pH 7.4 with ^{14}C(Bz) (final concentration 0.28 mM for proteins and lipids and 0.15 mM for DNA).

Blank values, expressed as pmol Bz bound/mg, were the following: 82 for proteins, 3345 for lipids, 55 for n-DNA and 615 for k-DNA. The data represent the average of two determinations. DNA is from a pool of two incubation mixtures, because of the scarcity of the material.

Data were expressed in pmol Bz/mg on the assumption that all the radioactivity was in the form of the unchanged drug.

Results. Incubation of *T. cruzi* epimastigotes with ^{14}C-Bz resulted in the formation of reactive intermediate (s) capable of binding to proteins, lipids and n- and k-DNA of the parasite (table). ^{14}C from Bz incorporation increased from 0.5 to 3 h, although linearity was rather poor.

When total lipids isolated from incubation mixtures were separated by chromatography into classes, more than 95% of the ^{14}C from Bz appeared in the phospholipid fraction, the rest being distributed among monoglycerides, diglycerides, triglycerides, free fatty acids, cholesterol and cholesterol esters. The pattern of ^{14}C radioactivity incorporation was similar at 0.5-, 1- and 3-h incubation times.

Covalent binding of Bz reactive intermediate (s) to k-DNA, unlike that to n-DNA, was only observed after 3-h incubation (table).

Discussion. These results show a significant metabolism of Bz by *T. cruzi* procyclic culture forms that resulted in activation of Bz and subsequent binding of reactive metabolites to DNA, proteins and lipids. These characteristics are similar to those reported for mammalian preparations[8-11]. Bz nitroreduction is mediated by NADPH cytochrome *c* reductase and cytochrome P-450 in liver microsomes[8-20]. Metabolic reduction of the drug by the parasite[6] could be ascribed to the same enzyme system[17-19]. These systems are, however, sensitive to oxygen. It is not known at present whether *T. cruzi* has nitroreductive activity mediated by xanthine oxidase or aldehyde oxidase as in the case of liver cytosol[8]. There are several potential reactive intermediates formed by enzymatic nitroreduction of Bz or other nitroimidazoles, such as the nitroanion radical[6] and the hydroxylamine or hydronitroxide radicals[1,2,8,20].

The interactions described here may explain, at least in part, the deleterious effects of Bz on *T. cruzi*, particularly its different mechanism of action compared with Nfx[1-6]. The interaction of Bz reactive metabolites with *T. cruzi* DNA, proteins and lipids could be related to the inhibitory effect of Bz on growth in cultures, inhibition of macromolecular synthesis and enhanced macromolecular degradation[3,21-23].

There is considerable evidence that in the case of mammals, covalent binding of reactive metabolites of chemicals to cellular components is relevant to decreased macromolecular synthesis, enhanced degradation and cell death[24-25].

We cannot discard, however, the possibility of other, still unknown, more specific interactions of Bz with enzyme systems of the parasite which might account for the toxicity of the drug.

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