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PREVENTION OF BENZNIDAZOLE-INDUCED PROLONGING EFFECT ON THE PENTOBARBITAL SLEEPING TIME OF RATS USING DIFFERENT THIOL-CONTAINING COMPOUNDS

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Abstract

Benznidazole (BZ) is a nitroimidazolic chemotherapeutic agent employed against the acute and indeterminate phase of Chagas' disease, a tropical sickness afflicting more than twenty million people in Latin America. BZ has serious toxic side effects forcing people to stop treatment. These effects were attributed to the nitroreductive metabolic activation of BZ to a hydronitroxide radical or the hydroxylamine, which would covalently bind to cellular components. One of these deleterious effects is the prolongation on the pentobarbital sleeping time of rats. This results from the covalent binding of BZ reactive metabolites, arisen during its nitroreductive metabolism, to the phospholipid component of the mixed function oxidase which biotransform the barbiturate.

In this study, the potential ability of different thiol containing drugs to trap BZ reactive metabolites and to prevent BZ effect on the pentobarbital sleeping time was tested. Our HPLC studies evidenced that cysteine, N-acetylcysteine, penicillamine and glutathione were able to trap BZ reactive metabolites *in vitro* to produce one or two adducts. Reduced lipoic acid instead, decreased the intensity of the nitroreductive process without leading to detectable adducts. The *in vivo* administration of the thiol drugs, at dosage regimes available in literature, was able to markedly prevent the BZ prolongation effect on the sleeping time. Whether these thiols might prevent other BZ toxic effects without harming its chemotherapeutic actions remains to be established.

Introduction

Benznidazole (BZ) (Fig.1) is a chemotherapeutic agent against the acute phase of Chagas' disease, an endemic sickness afflicting at least 20 million people in Latin America

(Castro and Toranzo, 1988; Docampo and Moreno, 1985). More recently, BZ has also found use in the so called indeterminate phase of the disease occurring between the end of the acute phase and the appearance of the electrocardiographic alterations, typical of the chronic phase of the disease (Sosa Estani and Segura, 1999).

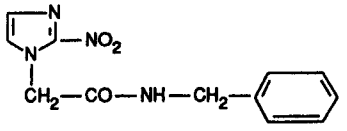


Fig.1: Benznidazole

BZ has evidenced in its clinical use several significant undesirable side effects which frequently force to stop treatment (Castro and Toranzo, 1988; Docampo and Moreno, 1985). In addition, BZ exhibited under experimental conditions relevant toxic effects including mutagenic and carcinogenic (Gorla and Castro, 1985; Nagel and Nepomnaschy 1983; Ohnishi, Ohashi *et al.*, 1980; Teixeira, Calixto *et al.*,1994) and deleterious actions in testes; ovaries or adrenales (Bernacchi, de Castro *et al.*, 1986; de Castro, Toranzo *et al.*, 1989, 1990, 1992). It was also able to prolong the pentobarbital sleeping time of rats apparently by inhibiting the liver microsomal transformation of the barbiturate at the mixed function oxigenase system (MFO) (Aguilar, Toranzo, *et al.*, 1990, Masana, Toranzo, *et al.* 1985). All these deleterious effects of BZ were found to be related to BZ nitroreductive biotransformation to reactive hidronitroxide free radical or hydroxylamine which covalently bind to macromolecules such as DNA, proteins and lipids (Fig. 2) (Castro and Toranzo, 1988).

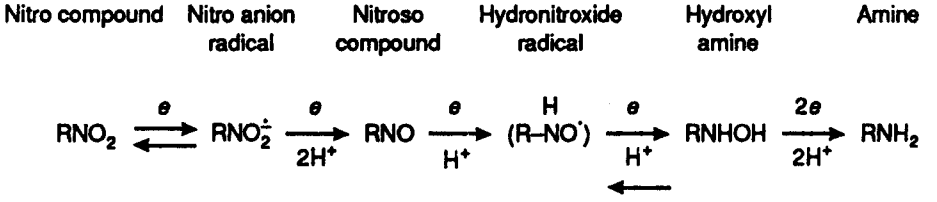


Fig. 2: Reductive metabolic bioactivation of BZ

Prior studies from others and from our laboratory evidenced that several thiol-containing molecules might effectively prevent chemically-induced cell injury and some of their manifestations *in vivo* by trapping reactive alterating moieties or free radicals (Bacq, 1965; Castro, 1980; Kemper, Jekat, *et al.*, 1990; Osborn, 1993; Prescott, 1990). In this

work, we tested the potential preventive effects of some clinically useful thiol containing drugs on the BZ induced prolongation effect on the pentobarbital sleeping time of rats as they relate to their ability to give adducts upon interaction with the BZ reactive metabolites.

Materials and Methods

Chemicals. Benznidazole (N-benzyl-2-nitro-1-imidazole acetamide) was a gift from F.Hoffmann La Roche and Company, Ltd . Enzymes, cofactors and thiol-containing compounds were purchased from the Sigma Chemical Co. (St. Louis, MO). All other chemicals employed were analytical grade.

Animals and treatments. Sprague-Dawley male rats (240-280 g) were used in the experiments. Food was withdrawn 12-14 h before use but water was available *ad libitum*. Temperature in the room was (23 ± 2) °C, the relative humidity was between 35 and 65% and the light was on from 6 AM to 6 PM. Sodium pentobarbital (8 mg/ml) was given ip in 0.9 % NaCl at a dose of 40 mg/kg. Benznidazole (BZ) was given po suspended in carboximethylcellulose 1% (CMC) (40 mg/ml) at a dose of 100 mg/kg. L-cysteine hidrochloride (CYS) was given po in distilled water (380 mg/ml) at a dose of 1.9 g/kg. Glutathione, reduced form (GSH) was given po in acidified distilled water (400 mg/ml) at a dose of 2.0 g/kg. DL-α-lipoic acid, reduced form (LIP) was given po in distilled water (40 mg/ml) at a dose of 100 mg/kg. N-acetyl-L-cysteine (NAC) was given po in distilled water (200 mg/ml) at a dose of 2 g/kg. Penicillamine (PEN) was given po in distilled water (180 mg/ml) at a dose of 1.8 g/kg.

For the *in vitro* studies animals were sacrificed by decapitation, livers were rapidly removed, weighted and processed.

Assay of the pentobarbital sleeping time. The sleeping time was recorded after the administration of pentobarbital to rats with BZ and the different thiol-containing compounds.

Isolation of microsomes. Livers were homogenized in 4 volumes of 1.15 % KCl in a teflon-glass Potter-Elvehjem homogenizer. The homogenates were centrifuged for 20 min at 9000 g and the supernatants were further centrifuged for 1 h at 105000 g to obtain the microsomal fraction. All these operations were performed at 2 to 4 °C.

Enzymatic and chemical determinations. Incubation conditions were essentially as described by Masana, Toranzo, *et al.* (1984) for bioreductive activation of BZ. All incubations were carried out in 20 ml rubber-stoppered sealed vials at 37°C. The vials, in a final volume of 2.5 ml of 20 mM potassium phosphate buffer (pH 7.4), contained: I) the cellular fraction suspended in phosphate buffer (final concentration from 9 to 10 mg protein/ml); II) 0.5 mM NADPH-generating system whose composition was 0.3 M Tris/HCl buffer (pH 7.4) 0.2 ml; 1 M $MgCl_2$ 0.2 ml; isocitric acid dehydrogenase type IV from porcine heart 0.6 ml; dl-isocitric acid trisodium salt 124 mg; NADP sodium salt 20 mg; III) BZ (final concentration 0.288 mM, except the GSH experiment were the final concentration of BZ was 0.144 mM) and IV) the sulfhydryl compounds in the incubation buffer (final concentration 7 mM). Vials containing cellular fraction suspensions were bubbled with oxygen-free nitrogen for 5 min prior to the addition of NADPH and/or BZ as a methanol solution. The final concentration of the methanol solution in the incubation mixture was 2 %. After 1 h of anaerobic incubation, reactions were terminated by the addition of 2 volumes (7.0 ml) of methanol. The suspensions were centrifuged for 45 min at 48000 g at 4 °C and an aliquot of the supernatants were further removed and filtrated through nylon filter membranes (pore size 0.45 μm) prior to HPLC analysis.

Protein concentration was determined with bovine serum albumin as a standard (Lowry, Rosebrough, *et al.* 1951).

High-performance liquid chromatography The determination of BZ content in the incubation mixtures was as follows. The biological sample (50 μl) was chromatographed at 30 °C using a Varian model 5000-CDS 401 Data System liquid chromatograph with a Supelcosil LC-18 column (25 cm x 4.6 mm I.D., 5 μm particle size). The mobile phase, consisting of 35 % methanol in 50 mM potassium phosphate buffer, pH 3.0, containing 5 mM octanesulphonic acid, was delivered at a constant flow-rate of 1.0 ml/min. The column effluent was monitored at 229 nm with a Varian UV 50 detector, 0.01 A.U.F.S., at that wavelength BZ and other compounds could be detected. Comparisons were made by peak-area ratio.

Results and Discussion

The here reported observations evidenced that different thiol-containing drugs having very well established use in both clinical and experimental conditions (Bacq, 1965;

Castro, 1980; Cotgreave, 1997; Ferreyra, Castro *et al.*, 1974; Ferreyra, Fenos *et al.*, 1977, 1979; Gorla, Ferreyra *et al.*, 1983; Kemper, Jekat *et al.*,1990; Osborn, 1993; Prescott, 1990; Valles, Castro *et al.*, 1993) were able to prevent *in vivo*, the BZ induced prolongation effect on the pentobarbital sleeping time (Table I).

TABLE I
EFFECT OF DIFFERENTS TREATMENTS ON PENTOBARBITAL SLEEPING TIMES OF RATS PRETREATED WITH BENZNIDAZOLE

TREATMENT[a]	PENTOBARBITAL SLEEPING TIME MIN ± SD	% OF CONTROL [b]
CONTROL	084 ± 08	100
BZ	290 ± 51	345
CYS	076 ± 32	090
CYS+BZ	135 ± 52	161
GSH	112 ± 03	133
GSH+BZ	115 ± 41	136
LIP	107 ± 11	127
LIP+BZ	170 ± 21	202
NAC	087 ± 12	103
NAC+BZ	139 ± 35	165
PEN	080 ± 04	095
PEN+BZ	126 ± 23	150

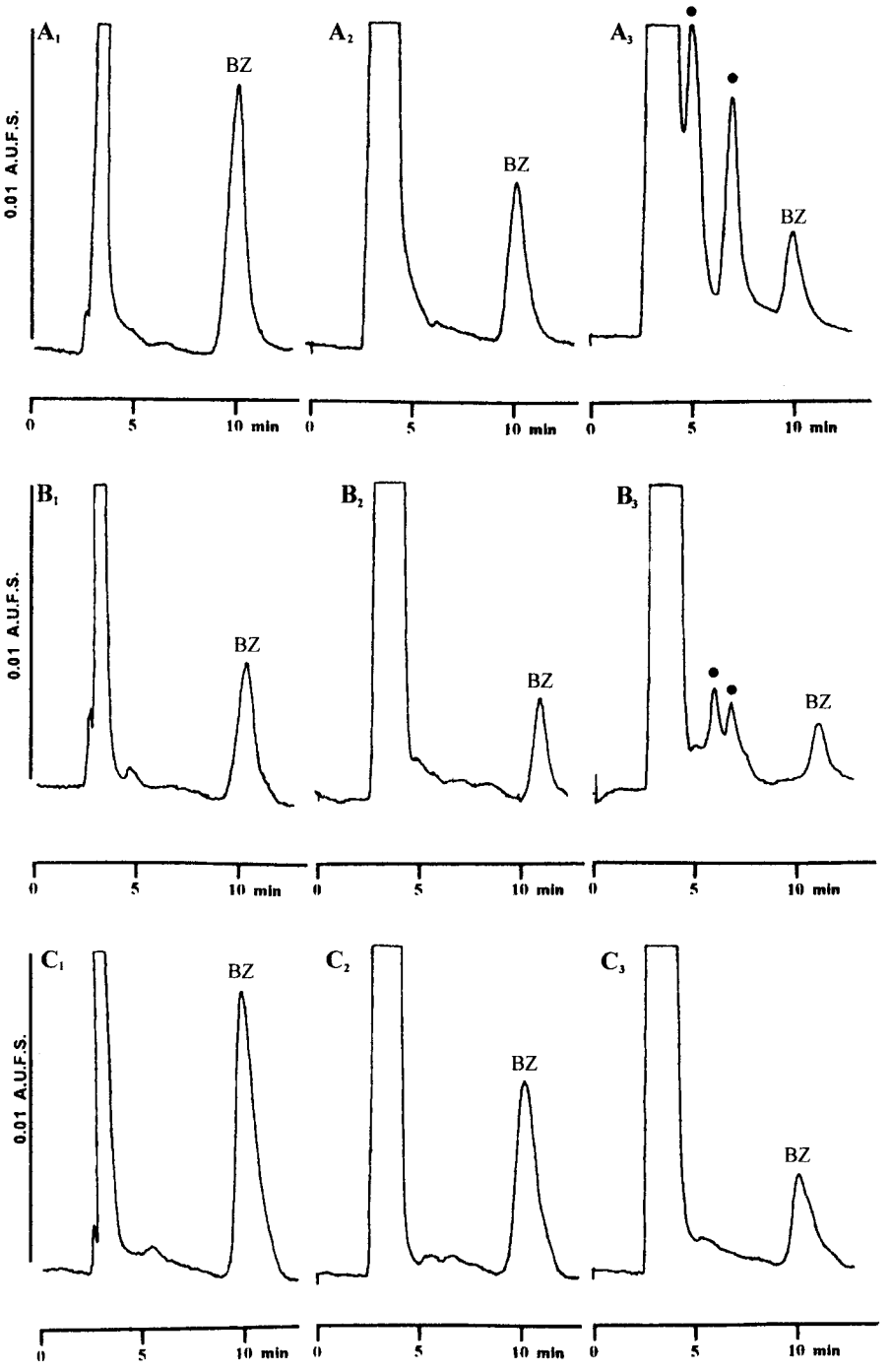
a-Sodium pentobarbital was given ip at a dose of 40 mg/kg to all the rats used in this experiment. The animals were fasted 12-14 h before the administration of the tested compounds but had free access to water during starvation. Six animals were used in each group.
The tested thiol-compounds were given po 90 min before pentobarbital. Controls received an equivalent amount of water.
Doses administered: CYS: 1.9 g/kg, GSH: 2 g/kg, LIP: 100 mg/kg, NAC: 2 g/kg, PEN: 1.8 g/kg
Sixty min before pentobarbital administration, BZ was given po at a dose of 100 mg/kg. Controls received an equivalent amount of CMC 1%.
b-The sleeping time of the respective control was taken as 100%

p>0.5	P<0.015	P<0.01	P<0.001
CYS vs CONTROL	[NAC+BZ] vs CONTROL	[CYS+BZ] vs CONTROL	BZ vs CONTROL
[GSH+BZ] vs CONTROL	[LIP+BZ] vs BZ	LIP vs CONTROL	GSH vs CONTROL
NAC vs CONTROL	[CYS+BZ] vs BZ	[PEN+BZ] vs BZ	[GSH+BZ] vs BZ
PEN vs CONTROL		[PEN+BZ] vs CONTROL	[LIP+BZ] vs CONTROL
			[NAC+BZ] vs BZ

At the dosage regime employed, those drugs were already known to be either efficient protective agents and/or preventive, against the acute toxicity of several poisons (Bacq, 1965; Ferreyra, Castro *et al.*, 1974; Ferreyra, Fenos, *et al.* 1977, 1979; Valles, Castro *et al.*, 1993). Their preventive effects in those already known cases, were attributed to their ability to trap reactive moieties (eg. free radicals, carbonium ions, alkylating

intermediates, metals, etc.) (Castro, 1980; Castro and Castro, 1997). Our HPLC studies where the BZ reactive intermediates (presumably a hydronitroxide free radical and/or a hydroxylamine) were produced by liver microsomal nitroreduction of BZ in the presence of NADPH (Castro and Toranzo, 1988; Masana, Toranzo *et al.*, 1984; Stoppani, 1999) revealed that in the presence of the preventive thiols, the total BZ concentration was always significantly reduced but more important, in most cases, the presence of adducts resulting from the interaction between the reactive metabolites and the thiol was detectable (Fig. 3). The only exception found was the case of reduced LIP, where the reduction in the level of BZ was not accompanied of formation of detectable adducts (Fig. 3: C_3). In this case, the reductive and H donating properties of LIP probably destroyed the reactive intermediates without leading to adducts. Both, hydronitroxide free radical or hydroxylamine, might be susceptible to be destroyed upon their reduction and/or H donation. We still do not know the precise structure of the resulting adducts. In the case of CYS, GSH and NAC, two adducts were neatly produced and they were more polar in nature than BZ itself (Fig. 3: A_3, B_3 and D_3). They might potentially reflect the reaction products of both BZ reactive moieties with the sulfhydryl group of the thiol drugs. In the case of PEN, however, only one adduct was detected and it was less polar than BZ. (Fig. 3: E_3). Potentially, the presence of two methyl groups in the PEN molecule replacing two hydrogens from CYS, favors the reaction of the SH groups with one of the reactive metabolites more that with the other. The presence of the methyl groups would also increase lipophilicity of the resulting adducts, as shown in the HPLC study of the reaction.

Of particular interest is the finding of the reaction between BZ reactive metabolites and GSH. On one hand, it opens the possibility for the use of this peptide as preventive agent but, more important, it also suggests that other GSH derivatives of more suitable nature, might also be employed as preventive agents. Furthermore, the positive results might also indicate, that a significant part of the preventive effects of CYS or NAC *in vivo*, might be due to their conversion to GSH (Cotgreave, 1997) and not merely because they also react with BZ reactive metabolites as evidenced by our *in vitro* HPLC experiments.


A1
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C1
C2
C3

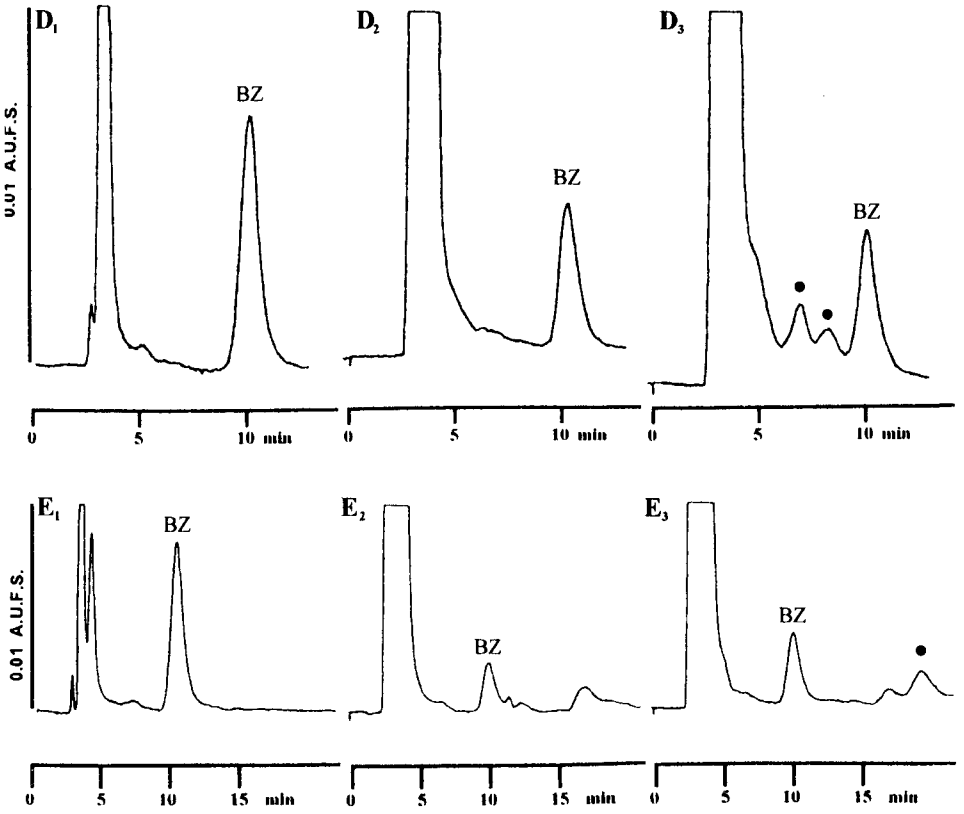

Fig. 3: HPLC chromatograms after anaerobic incubations of liver microsomal fraction containing: 1) BZ, 2) BZ and NADPH and 3) BZ, NADPH and the thiol-drugs. CYS(A), GSH (B); LIP (C), NAC (D) and PEN (D). Peaks: • = resulting adducts of BZ with thiol trapping agents. For details see *Materials and Methods.*

Irrespectively of all these theoretical considerations described above is the fact, that some clinically acceptable thiol-containig drugs were able to prevent a harmful manifestation of BZ toxicity *in vivo*. Whether other toxic effects of BZ might be equally prevented remains to be established. Further and far more important, it is critical to establish whether any use of some of these thiol drugs as coadyuvants of the BZ treatment of patients suffering Chagas' disease does not harm BZ chemotherapeutic effects of this compound against *T. cruzi.* Studies directed to answer these questions are in course in our laboratory.

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