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Original Research

Benznidazole-induced ultrastructural and biochemical alterations in rat colon[1]

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KEY WORDS benznidazole; benzene; nitroreductases; drug therapy; colonic diseases

ABSTRACT

AIM: To study the effects of benznidazole (Bz), a drug used in the chemotherapy of the acute and the intermediate phase of Chagas' disease, on the colon of rats. **METHODS**: Sprague Dawley male rats received Bz 100 mg/kg ig. After 24 h colons were examined by electron microscopy. Concentrations of Bz in colonic tissue were measured by HPLC. Bz nitroreduction was followed by the decrease in the drug concentration using spectrophotometry and HPLC or by covalent binding to proteins of reactive products formed under *in vivo* and *in vitro* conditions. **RESULTS**: Colon mucosa of Bz-treated rats showed intense ultrastructural alterations: abundant mucus secretion at the level of the Goblet cells and dilatation of the endoplasmic reticulum and the Golgi apparatus in epithelial cells. The concentration of Bz in tissue was (59 ± 18) and (93 ± 14) nmol/g (protein) 1 and 3 h after oral administration to rats, respectively. Colonic microsomes anaerobically activated Bz in the presence of NADPH. This activating nitroreductive pathway only involved a minor part of the total Bz and could not be detected spectrophotometrically or by HPLC analysis of the Bz consumed. Reactive metabolites that bound covalently to microsomal proteins were formed in this process. The covalent binding was also observed *in vivo* 1, 3, 6, and 24 h after administration of the labeled drug to rats. **CONCLUSION**: Reactive Bz metabolites produced during nitroreductive bioactivation of the drug in the colonic mucosa could interact with proteins and other cellular constituents to cause injury.

INTRODUCTION

Chagas' disease is an endemic sickness afflicting Latin American countries[1-3]. There are at least 20 million people suffering the disease and about 60 million living in areas of potential infection. Threatening effects on the heart and the central nervous system and intense inflammatory processes in the colon and the esophagus might occur after the acute phase of Chagas' disease[1]. Further, there are reports of significant increased incidence of colonic and esophageal cancers in chagasic patients[4-6].

Benznidazole (Bz) is a drug used in the treatment of the disease[1,2,7,8]. This therapeutic agent has serious side effects that frequently force physicians to stop treatment[9-11]. Recently, the use of Bz has been extended to the period after the acute and before the chronic phase of the disease, the so-called intermediate or indeterminate phase[7,12,13].

Bz needs a nitroreductive biotransformation to reactive metabolites, that bind covalently to macromolecules and lipids, to cause injury[8,10,11,14]. These deleterious effects might be additive or synergistic with those induced by the evolution of the disease. In this regard it was of interest to report whether epithelial colonic mucosa is able to reductively biotransform Bz to reactive metabolites that interact with colonic proteins and produce cell injury.

MATERIALS AND METHODS

Chemicals Bz (*N*-benzyl-2-nitro-1-imidazole ac-

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etamide) and Bz with ^{14}C in position 2 of the imidazole ring (sp act 2.15 TBq/kg) were gifts from F Hoffmann La Roche and Company, Ltd. No detectable-labeled impurities were found in the sample of [^{14}C]Bz that was analyzed by silica gel TLC using chloroform-ethanol (9:1) as solvent. All other chemicals employed were of analytical grade.

Animals and treatments Sprague-Dawley male rats 15-wk old of body weight 200 – 300 g were bred in our laboratory. Rats were group-housed, five rats per cage, and were maintained in a controlled room on a 12-h:12-h light:dark cycle (light phase 07:00 – 19:00) with free access to food and water. Temperature in the animal room was (23 ± 2) ℃ and the relative humidity was between 35 % and 65 %. Animals were fasted 12 – 14 h before treatment. Bz was given ig at a dose of 100 mg/kg suspended in 1 % carboxymethylcellulose (CMC). Control rats received only CMC. Animals were sacrificed at different times after Bz or CMC administration and the colons were rapidly excised and washed until any remaining feces were removed. The mucosal layers instead of the whole colon were used for studies of metabolism. They were carefully scrapped and pooled before processing. The operation was performed at 4 ℃.

In the *in vivo* studies on covalent binding of Bz reactive metabolites to cellular constituents, the animals received [^{14}C]Bz ig at a dose of 10 mg/kg (1.85 MBq/kg) suspended in CMC. The animals were sacrificed 1, 3, 6, and 24 h after Bz treatment.

Transmission electron microscopy Five male rats per group (control and Bz-treated animals) were anesthetized by diethyl ether 24 h after CMC or Bz administration. The abdomen was cut by a midline incision and the colon was exposed. After removing the entire colon, the specimen was immediately placed in chilled 2 % formaldehyde-2 % glutaraldehyde in cacodylic buffer 100 mmol/L containing 0.02 % $CaCl_2$, pH 7.4, and promptly cut longitudinally. The tissue immersed in the fixative solution was gently agitated a few times to remove fecal material. After adequate fixation, ten cubes (3 mm^3) per rat colon were washed with barbital buffer and postfixed with 1 % osmium tetroxide. Then, they were stained as a whole with uranyl acetate, dehydrated with graded ethanol, and vertically embedded in epoxi resin to ensure proper orientation for sectioning perpendicular to the plane of the luminal surface.

Sections 1 μm thick were stained with toluidine blue and examined with a light microscope in order to select epithelial areas for thin sectioning. Thin sections were cut with diamond knives, stained with uranyl acetate and lead citrate and examined in a Philips EM 300 transmission electron microscope[15-17].

Isolation of subcellular fractions Colon mucosa was homogenized in a teflon-glass Potter-Elvehjem homogenizer with 4 volumes of Tris/HCl 20 mmol/L buffer containing sucrose 0.25 mol/L, edetic acid 5 mmol/L, pH 7.4. The homogenates were centrifuged at 9000 × *g* for 20 min. The resulting supernatant was centrifuged at 100 000 × *g* for 1 h and the microsomal pellet and the cytosolic supernatant was recovered. The latter was dialyzed overnight against 2 L of Tris/HCl buffer 20 mmol/L, containing sucrose 0.25 mol/L, edetic acid 50 mmol/L, pH 7.4. All samples were handled at 4 ℃.

Determination of Bz content in colonic tissue Three animals per group were used. Colons from rats receiving a single dose of Bz ig were pre-washed to remove fecal material and then homogenized with saline solution (12 volumes per g) in a high speed cutting, dispersing and emulsifying Ultra Turrax T_{25} tissue homogenizer (IKA Werk Germany). Control rats were run simultaneously. The extraction of 1 mL of the homogenate with 6 mL of dichloromethane was carried out with an Extrelut® column. The solvent was evaporated to dryness under nitrogen and the residue was dissolved in 0.5 mL of mobile phase. The samples (10 μL) were chromatographed at 40 ℃ using a Hewlett Packard model 1090 Serie II liquid chromatograph with a Hewlett Packard ODS Hypersil column (20 cm × 2.1 mm ID, 5 μm particle size). The mobile phase, consisting of 60 % methanol-water, was delivered at a constant flow-rate of 0.2 mL/min. The column effluent was monitored at 320 nm. The efficiency of recovery of Bz added to a colonic homogenate was > 70 %. Calibration curve was prepared by analyzing 1 mL of homogenized sample spiked with known amounts of the compound (ranging from 1.0 to 3.0 mg/L). Plots of peak areas against concentration were linear over the concentration ranges studied (correlation factor 0.97). The detection limit was 0.5 mg/L using 1 mL homogenate and allowing a signal-to-noise ratio of 2.

Determination of Bz nitroreductase activity All anaerobic incubations were carried out in 20 mL-rubber sealed vials with agitation at 150 oscillations/min in a water bath at 37 ℃. The incubation mixtures were pregassed with oxygen free nitrogen for 5 min. Standard incubations contained: potassium phosphate buffer 20 mmol/L (pH 7.4); the microsomal fraction (final con-

centration; 4 g protein/L); NADPH-generating system 0.5 mmol/L that contained; Tris/HCl buffer 0.3 mol/L (pH 7.4) 0.2 mL, $MgCl_2$ 1 mol/L 0.2 mL, isocitric acid dehydrogenase type IV from porcine heart 0.6 mL, *dl*-isocitric acid trisodium salt 124 mg, and NADP sodium salt 20 mg, and Bz 0.288 mmol/L in *N*,*N*-dimethylformamide (DMF). In the cytosolic enzyme activity assay NADPH was omitted and replaced by the appropriate substrate. The aldehyde oxidase substrate *N*-methylnicotinamide and inhibitor menadione were added to give a final concentration of 2.5 mmol/L and 10 μmol/L, respectively. The xanthine oxidase substrate hypoxanthine and inhibitor allopurinol were added to give a final concentration of hypoxanthine 0.25 mmol/L and allopurinol 0.15 mmol/L . All determinations for cytosolic enzyme activity were carried out at protein 6 – 8 g/L.

In the spectrophotometric determination of microsomal Bz nitroreductase activity vials containing a final volume of 2.5 mL were incubated for 35 min. Reactions were terminated by the addition of 1 mL of 15 % zinc sulfate. Incubation mixtures were poured over 2.5 g of NaCl and extracted with 7 mL of ethyl acetate. The organic phase was read in a spectrophotometer at 315 nm. At this wavelength Bz has maximum absorption.

In the determination of Bz nitroreductase activity by HPLC, vials containing a final volume of 0.5 mL of microsomal (or cytosolic) suspension were incubated for 60 min. Reactions were stopped by the addition of 1 mL of methanol. The samples (1.5 mL) were centrifuged at 13 000 × *g* for 15 min and the supernatants were chromatographed by reverse-phase HPLC essentially as described above. Quantitation was by peak-area ratio with reference to standards treated identically.

In these *in vitro* studies the Bz nitroreductase activity was followed by substrate disappearance. Twenty-five rats were used in each experiment.

Covalent binding (CB) of Bz reactive metabolites to microsomal proteins was determined under *in vitro* and *in vivo* conditions. In the former, 0.288 mmol/L [^{14}C]Bz (sp act 12.1 kBq/mol) replaced cold Bz in the microsomal incubations. After 10 min, the reaction was stopped by the addition of trichloroacetic acid (TCA). The precipitated proteins were then washed thrice with 5 % TCA, heated with 5 % TCA for 30 min at 85 to 90 ℃ and washed successively, until radioactivity was negative, with ethanol-diethylether-chloroform (2:2:1), acetone, and finally with diethylether. In this experiment Bz reductase activity was followed by product formation. Microsomal fraction was obtained from six rats.

The same procedure was used when [^{14}C] from [^{14}C]Bz was determined in the microsomal colonic proteins *in vivo*. Three animals per group were used. Microsomes from rats receiving the labeled drug and sacrificed at different times, were isolated, washed, and dried as indicated above. In both cases radioactivity was assessed dissolving the samples in formic acid and counting them in a liquid scintillator.

Protein concentrations were determined using the method of Lowry *et al*[18] with bovine serum albumin as a standard.

RESULTS

Transmission electron microscopy The observation of the colonic mucosa of control rats showed that the wall of the colon did not have villi plicae circularis. It exhibited numerous folds with a typical structure of columnar lining epithelium, a core of lamina propia and the muscularis mucosa.

Most cells in the intestinal epithelium from control rats were tall columnar absorptive cells with striated borders (Fig 1). The cytoplasmic extensions forming the microvilli covered the apices of the intestinal absorptive cells (Fig 1).

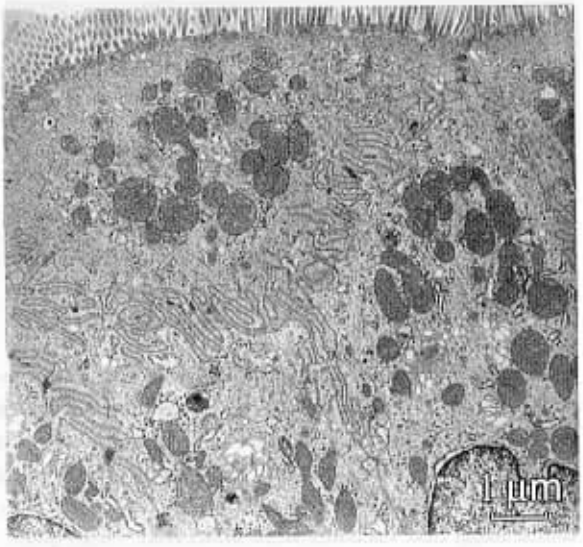

Fig 1. Transmission electron micrograph from columnar epithelial cells of colon of control rat. Numerous mitochondria and interdigitation folds of adjacent cell surfaces were present in the cytoplasm. The borders had numerous microvilli. Abundant free ribosomes and Golgi complexes were also observed. × 10 800.

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The effects of Bz on colonic tissue, observed 24 h after administration to rats, involved Goblet and epithelial cells. Multiple mucus-secreting globules occupying large areas of the cytoplasm gave evidence of hypertrophy of Goblet cells. The granules were bounded by a single membrane. Mature granules, of all forms and sizes, were large and electron-lucent (Fig 2A).

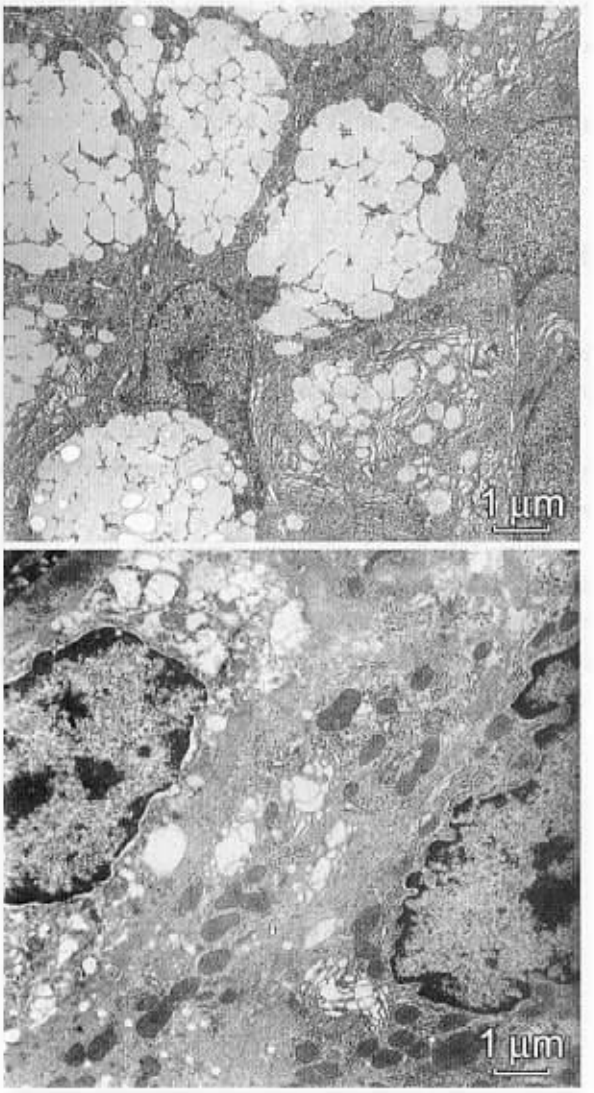

Fig 2. Electron micrograph from colon of benznidazole-treated rat. A, increased number of Goblet cells with closely packed mucigen granules or mucous droplets were shown. × 7000; B, convoluted nucleus showing deep indentation with large aggregates of chromatin, dilated Golgi and vacuoles were observed. × 10 800.

Epithelial cells exhibited irregular distribution and shortening of microvilli. The nucleus was often convoluted, showing deep indentations with denser peripheral chromatin and presence of a huge nucleolus and numerous vacuoles (Fig 2B). Cytologic epithelial changes included dilatation of the endoplasmic reticulum and the Golgi apparatus (Fig 2B).

Bz content in colonic tissue of rats Bz given ig was rapidly present in significant amounts in colon (Tab 1). Colon levels of Bz increased from 1 to 3 h after the administration.

Tab 1. Benznidazole (Bz) content in colonic tissue of rats at different times after administration. $n = 3$ determinations. $\bar{x} \pm s$.

Time after administration /h	Bz content/ nmol·g^{-1}(wet weight)
1	59 ± 18
3	93 ± 14

Bz nitroreductase activity in rat colon In the microsomal fraction no significant Bz nitroreductase activity in anaerobic conditions could be detected by spectrophotometric or by HPLC procedures. The results are from three independent spectrophotometric and HPLC determinations.

No nitroreductase activity was detected when the dialyzed cytosol fraction was incubated with the aldehyde oxidase substrate *N*-methylnicotinamide and the inhibitor menadione or the xanthine oxidase substrate hypoxanthine and the inhibitor allopurinol. The results are from three independent HPLC determinations of the aldehyde oxidase and the xanthine oxidase enzymatic activities.

However, reactive products were formed in anaerobic colonic microsomal incubations with [^{14}C]Bz and NADPH generating system. Covalent binding (calculated on the assumption that the whole radioactivity was in the form of the unchanged drug) of (5.7 ± 0.5) nmol·min^{-1}·g^{-1} protein was detected, despite its small intensity. Value represents the mean of three different determinations.

The presence of Bz nitroreductase enzymatic activity was also observed *in vivo* by the CB of [^{14}C] from [^{14}C]Bz to microsomal colonic proteins from rats sacrificed 1, 3, 6, and 24 h after administration of the drug (Tab 2).

DISCUSSION

The results obtained provide evidence that Bz given orally is able to partially reach as such the colonic mucosa (Tab 1). We found that the large intestine had Bz

Tab 2. ***In vivo*** **covalent binding (CB) of benznidazole (Bz) metabolites to microsomal proteins.**
$n = 3$ rats. $\bar{x} \pm s$.

CB of Bz/nmol·g^{-1}(protein)			
1 h	3 h	6 h	24 h
22.9 ± 0.9	52.6 ± 12.6	32.9 ± 11.2	14.5 ± 0.9

nitroreductase activity and that it was able to biotransform Bz to reactive metabolites (Tab 2). Our laboratory previously reported that reactive metabolites, presumably hydronitroxide radicals, were formed during nitroreductive Bz metabolism[10,14-17]. Cytochrome P-450 (P-450), P-450 reductase, xanthine oxidase (XO), and aldehyde oxidase (AO) were reported to participate in the process[14-17]. The colonic mucosa has cytochrome P-450 and cytochrome P-450 reductase in the microsomal fraction[19] and xanthine oxidase (XO) in the Goblet cells and in the mucus[20]. In these studies we could detect the microsomal but we failed to detect any cytosolic XO- or AO- mediated Bz nitroreductase activity. This failure might be due to the relative small proportion of Goblet cells in relation to the other types of cells in the colonic mucosa and to the dilution occurring during homogenization and preparation of the cytosolic fraction.

It was reported that free radical metabolites were subject to addition and H atom abstraction reactions[21]. In this study, the Bz-derived free radicals would lead to addition reactions for they covalently bind to colonic mucosa proteins (Tab 2). Bz reactive metabolites not only bind to proteins but also to lipids and DNA[10,14-17,22,23]. The toxic action of Bz exerted on other tissues such as liver, testes, ovaries, adrenals[14-17], and even the process involved in the Bz action on *Trypanosoma cruzi* could be mediated by these interactions[23]. Consequently, it is reasonable to believe that they might play a role in the deleterious ultrastructural effects observed in the colonic mucosa at the level of the Goblet and the epithelial cells.

In the case of the Goblet cells whose role is to secrete mucus to the lumen, the effects of Bz include a remarkable increase in the mucus secretory activity. The potential clinical consequences of this might vary from only slight discomfort to serious diarrhea derived from the excessive secretion of water and mucus[24]. These consequences, however, would not be particularly serious provided they are neither long lasting nor too intense.

In the case of the epithelial cells, the observed deleterious effects involved the shortening of their microvilli and the dilatation of the endoplasmic reticulum/Golgi apparatus (Fig 2B). These effects, of reversible nature, were not strong enough to compromise cell survival. They may however lead to alterations in the mucosal absorptive/secretory functions and produce inflammatory changes. An alteration in these functions which cause decreased water absorption or impaired formation, transport and excretion of solid stools might result in significant distress to Bz treated patients[24]. Both effects can also be related to the gastrointestinal side effects reported during the clinical use of the drug[10]. A further point of concern is the fact that intense colon inflammatory processes usually accompany the course of the Chagas' disease. The presence of megaesophagus and megacolon are frequent in the course of this parasitic infection[1,4-6].

The present use of Bz in the post-acute phase, the so-called intermediate or indeterminate phase of the disease[7,12,13] might increase the risk of adverse effects in chagasic patients. The occurrence of ultrastructural cell injury mediated by Bz reactive metabolites and the presence of inflammatory events already in progress due to Chagas' disease evolution might coexist with potentially harmful consequences. This possibility deserves further attention.

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苄硝唑诱导大鼠结肠超微结构和生化改变[1]

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