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ANTILEISHMANIAL ACTIVITY AND MODIFICATION OF HEPATIC XENOBIOTIC METABOLIZING ENZYMES IN GOLDEN HAMSTER BY 2(3)-TERT-BUTYL-4-HYDROXYANISOLE FOLLOWING INFECTION WITH *LEISHMANIA DONOVANI*

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Abstract—Butylated hydroxyanisole (BHA) inhibited the growth of *Leishmania donovani* promastigotes (strain AG83) *in vitro*. The inhibition was dose dependent: the concentration of BHA required for 50% inhibition of the rate of growth was 1 μg/mL. BHA also prevented growth of *L. donovani in vivo* in golden hamsters infected with *L. donovani*. In addition, the effect of BHA on several enzymes involved in the metabolism of xenobiotics both in uninfected animals and animals infected with *L. donovani* is reported.

The increase in leishmaniasis throughout the world to epidemic proportions has increased the urgency for new and more effective drugs. *Leishmania donovani* is the protozoan parasite that causes visceral leishmaniasis. Chemotherapeutic agents widely used for the treatment of leishmaniasis are organic antimonial drugs [1]. Recently, visceral leishmaniasis clinically resistant to antimony has been reported [2, 3]. 2(3)-tert-Butyl-4-hydroxyanisole (BHA†) is a known antioxidant food additive of relatively low toxicity to humans and other animals. It is known to protect animals from many chemically induced neoplasias [4]. This could be explained on the basis of induction of drug-metabolizing enzymes that may interrupt the neoplastic process. Aldernate *et al.* [5] had earlier demonstrated that BHA decreased growth and respiration of *Trypanosoma cruzi* by inhibiting the electron transport chain between NADH and cytochrome *b*.

Various xenobiotics have been reported to alter the activity of the drug-metabolizing enzyme systems. Metabolism of these compounds can be divided into two stages, the phase I enzymes introduce or expose functional groups on xenobiotic molecules which can subsequently be conjugated with hydrophilic moieties in reactions catalysed by phase II enzymes. The most important reactions in phase I are oxidations catalysed by microsomal mixed function oxidases like cytochrome P450 (Cyt. P450) and aminopyrene-*N*-demethylase, while Phase II includes enzymes such as glutathione-*S*-transferase (GST).

This study was undertaken to determine the inhibition of *L. donovani* growth both *in vitro* and *in vivo* in the golden hamster by BHA. The report also describes the effect of BHA on several liver enzymes involved in the metabolism of xenobiotics both in infected and uninfected animals.

MATERIALS AND METHODS

Chemicals. All the biochemicals were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.).

Parasites. *L. donovani*, strain AG83 (MHOM/IN/1983/AG83) was kindly given by Prof. A. N. Bhaduri, Indian Institute of Chemical Biology (Calcutta, India) and was maintained at the School of Life Sciences, Jawaharlal Nehru University (New Delhi, India) by passage every 8–10 weeks in golden hamsters. The *in vitro* AG83 strain was maintained on blood–agar slants [NaCl 0.6%, Agar 1.5%, peptone 2%, brain heart infusion 3.7%, glucose 2% and rabbit blood 3.3% (v/v), pH = 7.2]. *In vitro* promastigote growth was studied in M-199 supplemented with HEPES and 10% fetal calf serum (pH = 7.2). Cells were counted in a Neubuer hemocytometer. The results are means ± SD of four determinations.

Leishmaniasis in the hamster. We used an 8 day test for screening drug against leishmaniasis in the golden hamster [6]. Hamsters 50–70 g were infected intracardially (on day 0) with approximately 10^8 amastigotes in 0.2 mL of phosphate-buffered saline (pH 7.4) and treated with BHA (0.4 g/kg body wt/day) on day 3 through day 6 (total dose = 1.6 g/kg body wt). BHA was administered orally (in 20 μL of absolute alcohol). The corresponding controls received an equal volume of the vehicle. On day 7 the liver and spleen were removed. The smears were made and stained with Leishman stain [7]. The number of amistigotes/500 organ nuclei was counted under oil immersion and the number of amastigotes/organ cell nucleus × organ wt (mg) ×

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† Abbreviations: BHA, butylated hydroxyanisole; Cyt. P450, cytochrome P450; Cyt. b_5, cytochrome b_5; AH, aniline hydroxylase; EOD, ethoxycoumarine-*O*-deethylase; GST, glutathione-*S*-transferase.

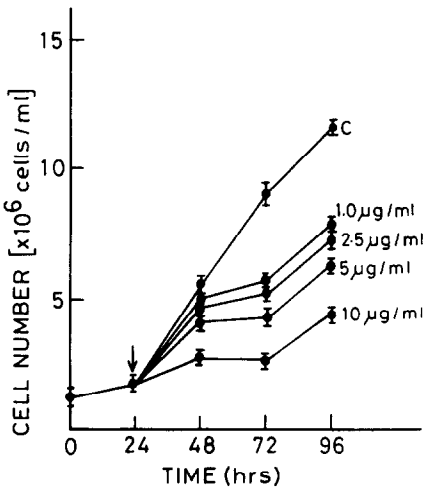

Fig. 1. Inhibition of growth of *L. donovani* promastigotes in the presence of different concentrations of BHA. Arrow indicates time of addition of the drug. Each point is the mean of four determinations ± SD.

2×10^5 = approximately total amastigotes/organs [8].

Enzyme determinations. The animals were killed 24 hr after the last treatment and starved overnight before being killed. Livers were homogenized after perfusion with 0.9% cold saline (a 25% homogenate was prepared) in 50 mM Tris and 50 mM KCl (pH 7.4), and centrifuged for 20 min at 11,000 *g* at 4°. The supernatant fraction was ultracentrifuged in a Beckman ultracentrifuge at 105,000 *g* for 1 hr and the microsomal pellet was resuspended in 50 mM Tris–HCl buffer pH 7.5. The cytosolic fraction (supernatant after ultracentrifugation) was kept at −70° for GST assay. All the other enzymes were assayed on the day of isolation. Cyt. P450 and cytochrome b_5 (Cyt. b_5) were determined by the method of Omura and Sato [9] using 92 $mM^{-1} cm^{-1}$ and 185 $mM^{-1} cm^{-1}$, respectively, as the molar extinction co-efficients. Determination of aniline hydroxylase (AH) was by the method of Nakanishi *et al.* [10]. Ethoxycoumarine-*O*-deethylase (EOD) was determined by the method of Lake *et al.* [11]. GST activity was measured by Habig's method [12] while acid-soluble sulfhydryl (−SH) levels were estimated as described by Moron *et al.* [13]. Protein was determined according to Lowry *et al.* [14]. Student's *t*-test was performed on the data and a probability value (P) of less than 0.05 was considered significant. Results are means ± SD of six to eight animals per group.

RESULTS

Figure 1 shows the antileishmanial activity of BHA *in vitro* against promastigotes. The highest dose 10 μg/mL of BHA resulted in substantial inhibition of growth. All other concentrations of BHA reduced the growth rate but did not stop growth. The LD_{50} for BHA was 1 μg/mL. The effect of BHA on parasite suppression in the liver and spleen following the treatment described above is shown in Table 1. In the spleen treatment with BHA resulted in 77% suppression of the parasite whereas in the liver there was a 66% inhibition of parasitemia. The effect of treatment with BHA on the microsomal enzymes (the mixed function oxidases) and GST and −SH levels in both infected and uninfected golden hamsters is shown in Table 2. BHA did not alter the microsomal protein or liver:body weight ratio (not reported).

Treatment with BHA resulted in a significant increase in several enzymes known to be involved in the oxidation of drugs in isolated microsomal fractions. A significant increase in the specific content of Cyt. P450 and Cyt. b_5 was observed in the uninfected control when treated with BHA. AH also showed a significant increase. However, there was no change in the activity of EOD and GST after treatment with BHA only. −SH levels also showed a significant increase over control values. Leishmania infection for 8 days did not cause a change in any of the enzymes except Cyt. b_5, where a significant increase ($P < 0.05$) was observed over control values. When the infected hamsters were treated with BHA, a significant decrease in Cyt. P450, EOD, SH and GST levels compared with the BHA only group was observed. However, no significant change in levels of Cyt. b_5 and AH activity was found compared with the BHA only-treated group.

DISCUSSION

The main purpose of this study was to investigate the antileishmanial activity of BHA both *in vivo* and *in vitro*, and its ability to modify the activity of some hepatic drug-metabolizing enzymes in the liver of both infected and uninfected golden hamsters. We found that BHA, an antioxidant and scavenger of

Table 1. Suppression of *L. donovani* at different anatomical sites in the golden hamster by BHA

Group	Organ	Total dose (g/kg)	Dose/day (g/kg)	No. of amastigotes ($\times 10^5$)*	% Infection	% Inhibition
Leishmania only	Spleen	0	0	28.5 ± 6.14	100	0
	Liver			984 ± 51.5	100	0
Leishmania + BHA	Spleen	1.6	0.4	6.54 ± 3.0	23	77
	Liver			330.4 ± 28.2	34	66

* Values are means ± SD.

Table 2. Effect of *L. donovani* infection and BHA on hepatic xenobiotic-metabolizing enzymes in hamsters

Groups	Cyt. b_5 (nm/mg protein)	Cyt. P450 (nm/mg protein)	AH (μm/min/mg protein)	EOD (relative fl/min/mg/ protein)	−SH (μm/g tissue)	GST (μm/min/mg protein)
Control	0.291 ± 0.037	1.091 ± 0.151	0.285 ± 0.04	1.84 ± 0.19	6.54 ± 0.248	8.51 ± 1.41
Leishmania only	0.367 ± 0.066*	1.186 ± 0.109‡	0.326 ± 0.099‡	1.91 ± 0.138‡	6.38 ± 0.715‡	7.98 ± 0.847‡
Leishmania + BHA	0.499 ± 0.075§	0.91 ± 0.2†	0.372 ± 0.055§	1.64 ± 0.33†	5.90 ± 1.17†	5.829 ± 1.43†
BHA only	0.5002 ± 0.099*	1.598 ± 0.148*	0.33 ± 0.027*	1.97 ± 0.07‡	7.67 ± 0.12*	8.524 ± 1.17‡

Values are means ± SD.
* Significant increase compared with control $P < 0.05$–0.001.
† Significant decrease compared with BHA only $P < 0.05$–0.001.
‡ Not significant compared with control.
§ Not significant compared with BHA only.

different reactive oxygen species, causes inhibition of *L. donovani* growth both *in vitro* and *in vivo* in golden hamsters. It has been demonstrated that BHA decreased the growth and respiration of *T. cruzi* by inhibiting the electron transport chain between NADH and cytochrome *b* [5]. BHA is also known to protect animals from many chemically induced neoplasias [4]. This latter effect could be explained on the basis of induction of drug-metabolizing enzymes that may interrupt the neoplastic process. Earlier reports indicate that treatment of mice with BHA resulted in a selective increase in the specific activity of AH and Cyt. b_5 [15]. In contrast, the activities of other microsomal mixed function oxidases were decreased [15] as reflected by the reduced activities of aminopyrine-*N*-demethylase and benzopyrine hydroxylase or by a lower content of cytochrome P450. In the case of the golden hamster we found that BHA caused a marked increase in cytochrome P450 content. The effect on the other mixed function oxidases and cytochrome b_5 content is in agreement with that observed in the mouse model [15]. Different animal species vary in drug metabolism following exposure to antioxidants. Divergence of the results can largely be explained in terms of dosage, differences in the sensitivities of the liver to the drug employed or route of administration, and finally widely recognised species differences in microsomal drug metabolism.

Earlier reports showed that infection of mice or hamsters with *L. donovani* impairs the host hepatic microsomal membrane capacity to metabolize xenobiotic compounds [16, 17]. It was observed that this decreased hepatic xenobiotic-metabolizing capacity results in altered drug clearance rates and so increases the risk of an adverse drug effect. However, the 8 day hamster model used in this study showed that short-term infection with Leishmania does not alter the hepatic xenobiotic-metabolizing enzymes. Treatment of these Leishmania-infected animals with BHA resulted in a decrease in Cyt. P450, EOD, −SH group and GST activities compared with those given BHA only. However, no change was observed in cytochrome b_5 content and AH. These results indicate that the decrease in hepatic xenobiotic-metabolizing enzymes may result in altered drug clearance rates in infected animals as compared with uninfected animals.

The results of this study demonstrate that *L. donovani* growth *in vitro* and *in vivo* in the hamster model could be inhibited by the phenolic food additive BHA. We also report the ability of BHA to alter the activities of drug-metabolizing enzymes in the golden hamster and also show altered drug metabolism in infected animals as compared with uninfected animals.

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