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Effects of *t*-butyl-4-hydroxyanisole and other phenolic antioxidants on tumoral cells and *Trypanosoma* parasites

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The antioxidant food additives 2(3)-*tert*-butyl-4-hydroxyanisole (BHA), 2,6-di(*tert*-butyl)-*p*-cresol (BHT) and the methyl and propyl esters of gallic acid inhibited *Trypanosoma cruzi* culture growth and oxygen consumption. The I_{50} values for growth and oxygen uptake with BHA were 0.284 and 0.400 and for BHT 0.083 and 0.235 mM, respectively. Moreover, BHA inhibited the respiration of several tumor cells, as well as of the procyclic and bloodstream trypomastigote forms of *T. brucei brucei*, with I_{50} in the range 0.29–0.52 mM. Inhibition of the parasites' oxygen uptake by BHA was not of the pure Michaelis-Menten type, but may be of a mixed form. It is postulated that these compounds are inhibitors because they resemble ubiquinone.

Respiration inhibition; *t*-Butyl-4-hydroxyanisole; (*Trypanosoma cruzi, Trypanosoma brucei,* Tumor cell)

1. INTRODUCTION

The trypanosomiases are health and economically important parasitic infections of man and domestic animals. *Trypanosoma brucei brucei* restrains the development of the livestock potential of Africa and *T. cruzi* is the protozoan parasite that causes Chagas' disease in America, affecting about 27 million people. Cancer is one of the most important causes of death in man. Chemotherapy for Chagas' disease [1,2] and cancer [3] is at present not satisfactory.

2(3)-*tert*-Butyl-4-hydroxyanisole (BHA), 2,6-di(*tert*-butyl)-*p*-cresol (BHT) and gallic acid esters are known antioxidant food additives of relatively low toxicity to humans and other animals. The LD_{50} values for rats and mice are of the order of 1.6 g/kg animal weight [4–6]. Recently we demonstrated that BHA decreased growth and respiration of *T. cruzi* by inhibiting the electron-transport chain between NADH and cytochrome *b* [7]. It is also known to protect animals from many chemically induced neoplasias [8]. This latter effect could be explained on the basis of an induction of drug-metabolizing enzymes that may interrupt the neoplastic process. Recently, Picardo et al. [9] demonstrated that BHA and other related chemicals inhibited the growth of several tumor cell lines in culture. The nature of this toxic effect was not explained [9].

We report the inhibition of *T. cruzi* growth and respiration by several phenolic food additives. BHA inhibited the respiration of not only trypanosomes but also of several tumor cell lines. We suggest that the inhibition effect of the compounds is based on their chemical structures, which mimic the reduced form of CoQ, ubiquinol.

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2. MATERIALS AND METHODS

2.1. *Materials*

Tryptose, tryptone and yeast extract were obtained from Difco. Fetal calf serum and all other chemicals were obtained from Sigma. BHA, BHT, gallic acid and its esters were recrystallized from ethanol-water.

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2.2. *Trypanosomes*

The strains Tulahuen, Y, and the clone Dm 28c of *T. cruzi* epimastigotes were grown at 28°C in Diamond's monophasic medium [7,10] with blood replaced by 4 μM hemin; the pH was adjusted to 7.2 before sterilization. Fetal calf serum was added at 4% final concentration. *T. cruzi* epimastigote growth was followed by nephelometry using culture flasks with a side-arm tube [7]. The parasites were harvested on the fifth day of growth by centrifugation at 500 × *g* for 10 min. They were washed twice with 0.17 M NaCl-0.052 M potassium phosphate (pH 7.5) and resuspended in Diamond's medium for oxygen uptake determinations.

T. brucei brucei procyclic trypomastigotes were cultured in a semi-defined medium as described by Cunningham [11]. The parasites were harvested after 2–3 days and resuspended in 60 mM phosphate buffer (pH 7.4), 7 mM NaCl, 1 mg/ml bovine serum albumin and 50 mM glucose for oxygen uptake determinations [12].

T. brucei brucei trypomastigotes were isolated from mice 2 days after intraperitoneal injection with 2×10^6 cells. Blood was collected through cardiac puncture and the trypomastigotes were separated from blood cells through a DEAE-cellulose column equilibrated with buffer containing 50 mM NaCl, 5 mM KCl, 70 mM glucose, 1 mg/ml bovine serum albumin and 50 mM bicine (pH 8.0) [12]. Isolated trypomastigotes were washed twice in the same buffer and suspended in the same media used for *T. brucei* procyclic trypomastigotes.

2.3. *Tumor cells*

Four different ascites tumor cell lines were employed: Ehrlich, 786A, TA3, AS30-D. The tumor cells were grown by intraperitoneal transplantation in animals and harvested after 7–9 days as described by Moreadith and Fiskum [13]. Ehrlich and 786A ascites cells were carried in 25–30 g male Swiss albino mice; TA3 tumor cells were maintained in 25–30 g CAF 1 Jax mice; AS30-D hepatoma cells were carried in 100–125 g female Sprague-Dawley rats. The cells were washed 3 times in a solution containing 150 mM NaCl, 5 mM KCl, and 10 mM Tris-HCl (pH 7.4) [13] and then resuspended in the same buffer plus 2.7 mM glutamine that was added for the oxygen uptake determinations.

2.4. *Cell respiration*

Oxygen uptake measurements were made polarographically with a Clark electrode no.5331 (Yellow Springs Instrument) in a YSI model 53 O_2 monitor linked to a 100 mV mono-channel Goerz RE 511 recorder. The volume of the chamber was 2.0 ml. Antioxidants were added in dimethyl sulfoxide. No effect of dimethyl sulfoxide was observed at the concentrations used.

3. RESULTS AND DISCUSSION

Table 1 lists the effect of BHA on the oxygen uptake of tumor cells and trypanosome parasites. The concentration of BHA required to inhibit 50% of the respiration in all organisms tested was in the range 0.29–0.52 mM.

Tumor cells, *T. cruzi* and the culture procyclic trypomastigotes of *T. brucei* have an ADP-phosphorylating cytochrome-based respiratory chain [14–17]. The bloodstream forms, trypomastigotes of *T. brucei* are completely dependent on glycolysis for their energy supply, since they do not have a cytochrome respiratory chain [14]. They utilize a glycerol-3-phosphate oxidase system (GPO) to shuttle electrons to molecular oxygen as a unique way to reoxidize the glycolytically produced NADH. *T. brucei* trypomastigotes utilized oxygen at a rate about 10-times higher than their cultured procyclic forms; nevertheless, the respiration of both was inhibited by very similar concentrations of BHA (table 1).

T. brucei GPO consists of two components: a glycerol-3-phosphate dehydrogenase and the oxidase part which are linked via ubiquinol [14,15]. Esters of the 3,4-dihydroxybenzoic acid and *p*-alkyloxybenzhydroxamic acids are effective inhibitors of the GPO [18,19] possibly because they resemble ubiquinol, thus binding to the ubiquinol

Table 1

Effect of BHA on tumor cells and on trypanosome parasites' respiration

	Control (ngatom O/min per mg protein)	I_{50} (mM)
Tumor cells		
TA3	8.75 ± 1.28	0.46
786A	6.31 ± 0.80	0.52
Ehrlich	5.27 ± 0.47	0.29
AS30D	8.35 ± 1.10	0.31
Trypanosomes		
T. brucei b. procyclic trypomastigotes	33.15 ± 2.03	0.31
T. brucei b. trypomastigotes	229.4 ± 9.5	0.40
T. cruzi epimastigotes Tulahuen strain	39.95 ± 2.44	0.40
T. cruzi epimastigotes LQ strain	38.93 ± 5.19	0.35
T. cruzi epimastigotes clone DM 28 c	36.75 ± 4.96	0.40

I_{50} values represent the concentration of BHA required to inhibit 50% of oxygen uptake. They were calculated from curves of oxygen uptake vs concentration of BHA. Control data are expressed ± SD; $n = 5$. Oxygen uptake of tumor cells was determined at 25°C and at a cell concentration equivalent to 2.5 mg protein/ml. *T. cruzi* respiration was determined at 28°C and using a cell concentration equivalent to 2 mg protein/ml. *T. brucei* respiration was measured at 25°C with a cell concentration equivalent to 1.5 mg protein/ml. See text

Table 2

Effect of antioxidants on *T. cruzi* respiration and culture growth

	I_{50} (mM)	
	Respiration	Culture growth
BHA	0.400	0.284
BHT	0.235	0.083
Gallic acid	>40.00	0.996
Gallic acid methyl ester	29.30	0.693
Gallic acid propyl ester	6.69	0.419
Ascorbic acid	no inhibition	no inhibition

I_{50} corresponds to the antioxidant concentration needed to inhibit 50% of respiration and culture growth, respectively. Data were calculated from curves of oxygen uptake and cell growth vs antioxidant concentration (n = 4). See section 2. Epimastigotes of the Tulahuen strain were used

receptor on the oxidase component of the GPO [18,19].

BHA also resembles structurally ubiquinol and inhibited the respiration of *T. cruzi* epimastigotes by inhibiting the cytochrome-based electron-transport chain at a point between NADH and cytochrome *b* [7]. In other experiments (not shown) BHA, BHT and the methyl and propyl esters of gallic acid also inhibited the respiratory chain of *T. cruzi* and tumor cells at the same point.

Table 2 shows the effect of several antioxidants upon the respiration and culture growth of *T. cruzi* epimastigotes. There is a good correlation between the inhibition of respiration and of culture parasitic growth by the phenolic antioxidants. Ascorbic acid did not inhibit but rather favoured parasite growth. BHA and BHT are the best inhibitors followed by the propyl and methyl ester of gallic acid.

The available evidence [7,12,18,19] plus the data in tables 1 and 2 support the hypothesis that BHA and the chemically related phenolic antioxidants, such as BHT and the gallic acid esters, interfere with the ubiquinone/ubiquinol-mediated electron transport in tumor cells and in trypanosomes. These compounds mimic ubiquinol and may bind to ubiquinol receptors in the cytochrome-based respiratory chain, either between complex I and complex III or between the dehydrogenase and the oxidase component of the GPO. Thus, the mechanism of inhibition appears to be the same as

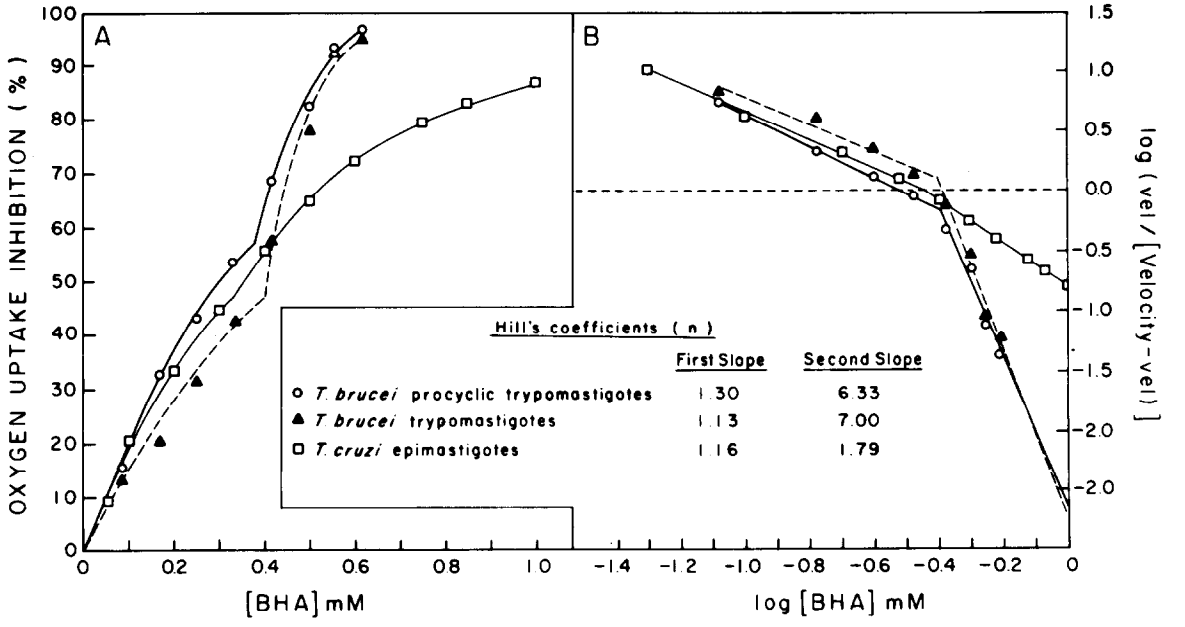

Fig.1. Inhibition of trypanosomes' respiration at different concentrations of BHA. (A) Oxygen uptake inhibition vs BHA concentration. (B) Hill plot of respiration inhibition by BHA. Velocity corresponds to non-inhibited oxygen uptake rate; vel. to the inhibited respiration rate. Linear regression coefficients all greater than 0.970. See text.

that proposed by Grady et al. [18,19] for the inhibition of the GPO in *T. brucei*.

Fig.1 shows the inhibitory effect on respiration of trypanosomes by BHA. Fig.1A is a direct plot of percentage inhibition vs BHA concentration. It is possible to appreciate that the inhibition does not follow a Michaelian hyperbola.

Fig.1B shows the same data on a Hill plot according to Monod et al. [20]. In the three cases under study curves with two different slopes are observed. The first part, those at the lower BHA concentration, has a Hill coefficient near unity, thus indicating that the inhibition follows a Michaelian model. The second part, at the higher BHA concentration, has a Hill coefficient greater than unity (inset, fig.1), thus indicating a cooperative effect. Many hypotheses may be put forward to explain the kinetics and mechanisms of this inhibition. At present the only feature that can be disproved is that the enzyme kinetics are not of the pure Michaelis-Menten type, but may be of a mixed form. Probably, there is also more than one binding site and rather good cooperativity.

Our results support the idea that these phenolic antioxidants act by mimicking coenzyme Q.

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