Pergamon

Biochemical Pharmacology, Vol. 47, No. 4, pp. 611–615, 1994.

0006-2952/94 $6.00 + 0.00

0006-2952(93)E0025-3

EFFECT OF ANTIOXIDANTS ON THE GROWTH AND POLYAMINE LEVELS OF *LEISHMANIA DONOVANI*

RITA MUKHOPADHYAY and R. MADHUBALA*
School of Life Sciences, Jawaharlal Nehru University, New Delhi-110 067, India

(*Received* 3 *December* 1992; *accepted* 26 *October* 1993)

Abstract—Butylated hydroxyanisole (BHA), retinoic acid (RA), retinol acetate (RAc) and sodium selenite (Na_2SeO_3) inhibited the growth of *Leishmania donovani* promastigotes (strains UR6 and AG83). There is a dose dependent inhibition of promastigote growth in both the strains. The concentrations of BHA, RA/RAc and Na_2SeO_3 required for 50% inhibition of the rate of growth were 0.5 μg/mL, 0.5 μM and 0.125 mM, respectively, for UR6. In the case of AG83, LD_{50} for BHA was 1 μg/mL whereas LD_{50} for RA/RAc and Na_2SeO_3 were the same as that of UR6. In *Leishmania spp.*, growth appears to be related to and dependent upon polyamine biosynthesis (Bachrach U *et al.*, *Exp Parasitol* **48**: 457–463, 1979). Experiments to test the possibility that these antileishmanial agents exert their inhibitory effect by blocking polyamine biosynthesis suggest that decrease in ornithine decarboxylase activity and the inhibition of polyamine levels could be a mechanism of inhibition of promastigote growth by BHA and RA.

Key words: antioxidants, *Leishmania donovani*; polyamines; antileishmanial

Protozoan parasites of the genus *Leishmania* are the causative agents of human Leishmaniasis, a widespread tropical disease transmitted by phelobotomine sandflies. Leishmaniasis manifests as a mild cutaneous lesion, mucocutaneous disease or fatal visceral form depending upon the species of the parasites [1, 2]. Currently, organic antimonial drugs are the only chemotherapeutic agents widely used for treatment of Leishmaniasis [2, 3] and it is generally agreed that better agents are urgently needed.

Some antioxidants, including vitamin A and its derivatives (retinoids) have been shown to play a critical role in cellular growth, differentiation and proliferation [4]. BHA†, a synthetic phenolic antioxidant and selenium an essential trace element for mammalian species and a potent antioxidant are known to suppress cell proliferation [5–8]. The effects of retinoids, BHA and selenium on cell proliferation in normal and neoplastic cells have been studied extensively and numerous changes in the proliferation pattern have been reported [8–14].

Retinoids have been shown to prevent induction of ornithine decarboxylase activity, the rate limiting enzyme in polyamine biosynthesis [15]. Kozumbo *et al.* [8] have reported that BHA strongly suppressed the TPA enhanced ODC activity, an initiator for skin tumor induction [16] and hyperproliferation [17].

The naturally occurring polyamines putrescine, spermidine and spermine are organic cations widely distributed in both procaryotic and eucaryotic organisms, including parasitic species e.g. trypanosomes [18] and *Leishmania* [1, 19]. In *Leishmania* as in other organisms, the levels of putrescine, spermidine and spermine fluctuate during the growth cycle [1]. Growth appears to be dependent on polyamine biosynthesis. This being the case we thought that antioxidants which are known to inhibit cell proliferation may do so by inhibiting the polyamine biosynthesis and have antileishmanial activity.

Here, we describe the inhibitory effect of retinoids, butylated hydroxyanisole and selenium on *Leishmania*. We have also studied the possible mechanisms of the inhibitory effect on Leishmanial promastigotes.

MATERIALS AND METHODS

Chemicals. All the biochemicals were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Fetal calf serum was obtained from Biological Industries (Israel). [^{14}C]Ornithine (51.6 mCi/mM) was from New England Nuclear (Boston, MA, U.S.A.). Precoated TLC plates were obtained from Anchrom Industries (Bombay, India).

Parasites. L. donovani strain UR6 (MHOM/IN/1978/UR6) and strain AG83 (MHOM/IN/1983/AG83) were kindly given by Prof. A. N. Bhaduri (Indian Institute of Chemical Biology, Calcutta, India) and were maintained at the School of Life Sciences, Jawaharlal Nehru University (New Delhi, India). The maintenance of promastigotes and growth inhibition studies in liquid media were done as described in Ref. 20. For polyamine estimation in *Leishmania donovani* (strains, UR6 & AG83) promastigotes were grown in α-MEM medium originally described by Kar *et al.* [21]. Since α-MEM is a completely defined medium, it contained no putrescine or polyamines. The promastigotes were grown at 22 ± 1°.

* Corresponding author. FAX (91) 011-686-5886.

† Abbreviations: BHA, butylated hydroxyanisole; RA, retinoic acid; RAc, retinol acetate; Na_2SeO_3, sodium selenite; DTT, dithiothreitol; ODC, ornithine decarboxylase; TPA, 12-*O*-tetradecanoyl phorbol-13-acetate.

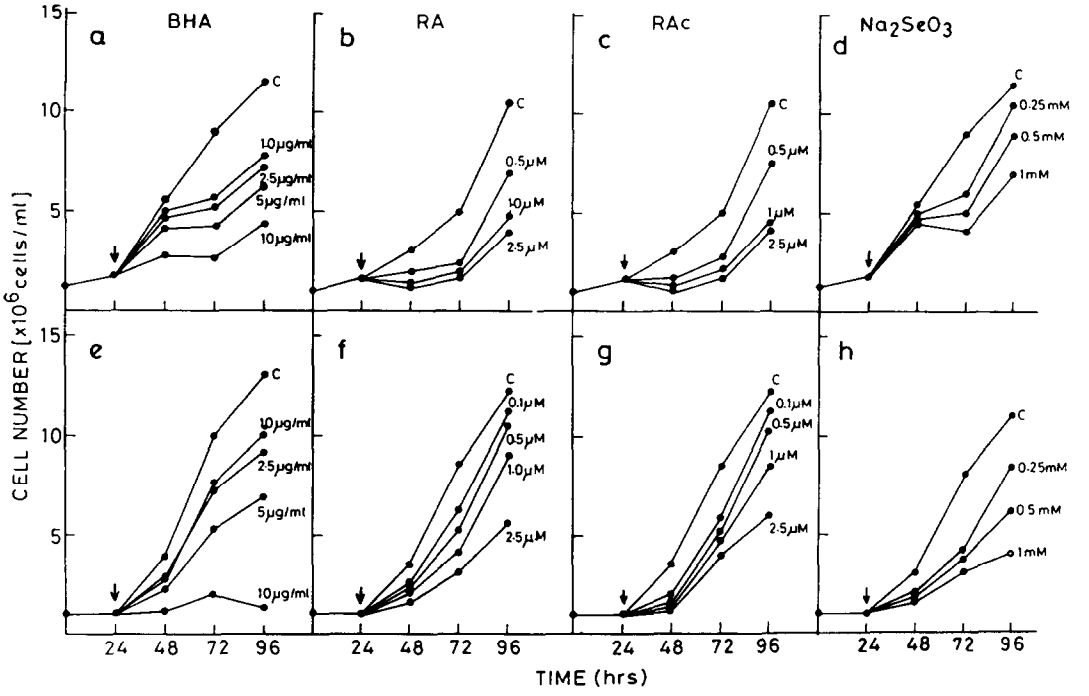

Fig. 1. Inhibition of growth of *Leishmania donovani* promastigotes (UR6 and AG83) in the presence of different concentrations of the antioxidants (a–d) AG83; (e–h), UR6. C, control, treatment with vehicle alone. Arrow, indicates the time of addition of the drug.

Drug study. BHA, RA and RAc were dissolved in ethanol and Na_2SeO_3 was dissolved in autoclaved distilled water and filter sterilized before addition to the medium containing parasites. The toxicity of the various drugs was determined by adding the drugs to a suspension of promastigotes (10^6 cells/mL) and incubating for 20 hr at 22°. The minimal concentration of the drug causing the inactivation of approximately 50% of the parasites within 20 hr was designated as LD_{50}. For growth studies, parasites were inoculated into the growth medium at a density of 10^6 cells/mL and drugs were added 24 hr later. The control group was treated with the vehicle.

Polyamine estimation. Cells (UR6 and AG83) were grown overnight free of drug to ensure exponential growth. Parasites were then inoculated in α-MEM at a density of 10^6 cells/mL and BHA (5 µg/mL), RAc (1 µm) and Na_2SeO_3 (0.5 mM) were added at 48 hr. Cells (10^7) were withdrawn at 72 hr time interval and assayed for polyamines. *Leishmania* promastigotes were sedimented by centrifugation at 1000 *g* for 10 min, washed with PBS (pH 7.4) and resuspended in 2% perchloric acid and kept overnight at 4° to extract polyamines. The dansyl derivatives were prepared according to Seiler [22] and separated by TLC on 0.2 mm thick silica gel on precoated plates with ethylacetate : cyclohexane (2:3) as solvent. Quantification of plates was done by a TLC scanner (TLC scanner II/CAMAG with CATS3/TLCII software programme). Unknown samples were compared to that of known standards.

ODC activity. ODC was measured according to Seeley [23]. Briefly, 2 mL suspension of promastigotes from a start culture of 3×10^6 cells/mL were taken and kept at 22°. BHA (5 µg/mL), RAc (1 µM) and Na_2SeO_3 (0.5 mM) were added at 48 hr and 2 mL samples were withdrawn at 72 hr and assayed for ODC. The promastigotes were washed twice with PBS (pH = 7.4) and finally suspended in 250 µL of harvest buffer (Tris–50 mM, EDTA 10 µM, DTT 2.5 mM, pH 7.5). The cells were freeze thawed twice before the actual assay. The ODC activity is expressed as nmol of CO_2 released per 10^7 cells. At least two aliquots of each sample (in duplicate) were analysed for the above estimations. The results are expressed as means ± SD of four determinations for each sample. Statistical analysis was done by Student's *t*-test.

Table 1. Growth inhibition of *Leishmania donovani* promastigotes *in vitro* by antioxidants

Drugs	Antileishmanial activity UR6 (LD_{50})*	AG83 (LD_{50})*
BHA	0.5 µg/mL	1 µg/mL
RA	0.5 µM	0.5 µM
RAc	0.5 µM	0.5 µM
Na_2SeO_3	0.125 mM	0.125 mM

* LD_{50} represents the concentrations of drugs which cause inhibition by 50% when compared to untreated controls.

RESULTS

Figure 1 shows that all four antioxidants namely

Table 2. Polyamine levels of *Leishmania donovani* promastigotes (strains UR6 and AG83) when treated *in vitro* with the antioxidants

Drugs	Dose	UR6 Putrescine (nM/10^7 cells)‡	UR6 spermidine (nM/10^7 cells)‡	Ag83 Putrescine (nM/10^7 cells)‡	Ag83 spermidine (nM/10^7 cells)‡
Vehicle	0	19.51 ± 3.59	19.03 ± 0.933	18.81 ± 0.622	35.27 ± 2.08
BHA	5 μg/mL	12.07 ± 1.48*	13.95 ± 1.29*	16.06 ± 0.806*	31.075 ± 1.87*
RAc	1 μM	9.492 ± 0.187*	12.81 ± 0.309*	9.8 ± 0.622*	23.14 ± 0.679*
Na_2SeO_3	0.5 mM	16.8 ± 1.48†	18.76 ± 1.18†	19.34 ± 0.12†	30.18 ± 1.856*

* Significant decrease over vehicle ($P < 0.05$–0.001).
† Not significant.
‡ Mean ± SD.

BHA, RA, RAc and Na_2SeO_3 exhibit anti-leishmanial activity against the promastigotes of two strains of *Leishmania donovani*—UR6 and AG83. The effect of BHA was greatest against UR6 where 10 μg/mL caused complete inhibition of growth (Fig. 1). In the case of AG83, the same concentration of BHA (10 μg/mL) was not completely inhibitory. All other concentrations of BHA reduced the growth rate but did not stop growth. RA and RAc were quite active against the promastigotes of UR6 and AG83 (Fig. 1). The percentage survival of UR6 and AG83 48 hr after addition of RAc was 47 and 25%, respectively, RA and RAc were both less effective than BHA in causing growth inhibition of promastigotes. Na_2SeO_3 when added to the growth medium, was found to be more effective against UR6 than AG83 (Fig. 1). The percentage survival of UR6 and AG83 48 hr after addition of Na_2SeO_3 was 36 and 66%, respectively. In both the strains, 1 mM concentration of Na_2SeO_3 reduced the growth rate but did not stop growth. In AG83 selenium was most effective in the mid log phase of the parasite. Table 1 shows the LD_{50} values of the four antioxidants. All the antioxidants tested were potent inhibitors of *Leishmania donovani* growth.

Retinoids and BHA are known to result in growth inhibition of various tumors in model systems and exert an antiproliferative effect on a variety of cell lines [24–29]. At the biochemical level they have been shown to markedly reduce the cellular level of ODC and polyamine [15]. Since a correlation between growth inhibition by these antioxidants and decrease in ODC activity and polyamine levels has been reported in various cell lines [15, 29], we postulated that these antioxidants inhibit *L. donovani* growth by interfering with polyamine biosynthesis. To test this possibility we studied the effect of RAc (1 μM) BHA (5 μg/mL) and Na_2SeO_3 (0.5 mM) on polyamine levels of *Leishmania* promastigotes (strains UR6 and Ag83). Table 2 shows the effect of BHA, RAc and selenium on the levels of polyamine in the *L. donovani* promastigotes. BHA and RAc resulted in significant inhibition of putrescine and spermidine levels in both the strains. Selenium did not result in any significant inhibition of spermidine and putrescine in UR6 and putrescine in the AG83 strain. A significant decrease in spermidine in AG83 strains ($P < 0.05$) was observed with selenium.

Retinoids and BHA are known to inhibit the growth of various tumor model systems [8–14] and this growth inhibition was correlated with a decrease in ODC activity [8, 17, 18]. This prompted us to study if the growth inhibition by these antioxidants resulted in *Leishmania* promastigotes correlated with the decrease in ODC activity. Table 3 shows the effect of RAc, BHA and Na_2SeO_3 on ODC activity of Leishmanial promastigotes (strains UR6 and Ag83). ODC activity was estimated as mentioned in Materials and Methods. BHA (5 μg/mL) resulted in 61% inhibition, RAc (1 μM) 41% inhibition and Na_2SeO_3 (0.5 mM) 26% inhibition in UR6 strain. Maximum inhibition of ODC activity was observed

Table 3. ODC activity of *Leishmania donovani* promastigotes (strains UR6 and AG83) when treated *in vitro* with the antioxidants

Drugs	Dose	UR6 ODC activity (nm/10^7 cells/hr)‡	UR6 % Inhibition	AG 83 ODC activity (nm/10^7 cells/hr)‡	AG 83 % Inhibition
Vehicle	0	0.65 ± 0.19	0	2.56 ± 0.17	0
BHA	5 μg/mL	0.25 ± 0.071*	61	1.45 ± 0.13*	43
RAc	1 μM	0.386 ± 0.05*	41	0.955 ± 0.301*	63
Na_2SeO_3	0.5 mM	0.48 ± 0.06†	26	1.105 ± 0.29*	57

* Significant decrease over vehicle ($P < 0.05$–0.001).
† Not significant.
‡ Mean ± SD.

with BHA. The inhibition observed with selenium was not significant over the control values, whereas in the Ag83 strain inhibition of ODC activity with BHA, RAc and Na_2SeO_3 was 43, 63 and 57%, respectively. However when the direct effect of the antioxidants was tested on the *Leishmania* ODC activity it was observed that BHA (5 μg/mL), RAc (1 μM) and Na_2SeO_3 (0.5 mM) caused significant inhibition of ODC activity (data not shown).

DISCUSSION

Antioxidants like BHA, retinoids and selenium are known to have antiproliferative effects [4–8]. Vitamin A and its analogs (retinoids) are known to induce a variety of biological and biochemical effects which seem to vary according to the cell type. Among these are their ability to prevent the induction and growth of tumors in model systems, to induce differentiation in a variety of cell lines and to exert an antiproliferative effect in certain cell lines.

The important role of reactive oxygen and free radical species in skin tumorigenesis has been much discussed [23, 24] and from this standpoint the effects of some antioxidants in suppressing tumor promotion have been studied with food additives like BHA and butylated hydroxytoluene (BHT) [8, 25–27]. Selenium, another potent antioxidant and an important trace element of mammalian diet has recently been reported to control human malaria if used in combination with vitamin E [28]. Since these antioxidants had a role in inhibiting cell proliferation we were prompted to study their effect on Leishmanial promastigote growth and to work out the mechanism of inhibition. All four antioxidants used, RAc, RA, BHA and selenium resulted in dose dependent inhibition of both the strains of *Leishmania donovani*, i.e. UR6 and AG83.

At the biochemical level retinoids have been shown to markedly reduce the cellular level of ODC and intracellular levels of polyamines [15]. Kozumbo *et al.* [8] have determined that BHA strongly suppresses TPA-induced ODC activity in the mouse skin. Taniguchi *et al.* [29] recently showed that the BHA induced inhibition of TPA induced ODC in the mouse skin is due to decrease in the ODC gene expression. These studies prompted us to study if the inhibition caused by antioxidants on *Leishmania* growth observed here is due to inhibition of polyamine synthesis levels and ODC activity. It was interesting to note that BHA and RAc resulted in significant inhibition of ODC in the promastigotes of *Leishmania donovani* (strains UR6 and AG83) and also inhibited the levels of putrescine and spermidine. On the other hand Na_2SeO_3 did not significantly decrease ODC activity in the strain UR6 nor polyamine levels in UR6 whereas in AG83 strain selenium caused a significant decrease in ODC activity and spermidine levels.

From these observations it is possible that retinoids and BHA induced growth inhibition of *Leishmania* may be due to depletion of polyamine levels. Growth inhibition by selenium shows a different pattern in both the strains. It is quite likely that selenium inhibition of leishmanial promastigotes may also involve some other mechanism.

The *in vivo* murine model is being used in this laboratory to check further the efficacy of these drugs. It will be interesting to explore the possibility of using these antioxidants in combination with some polyamine biosynthetic inhibitors.

Acknowledgement—Rita Mukhopadhyay is a recipient of a Research Fellowship from the University Grants Commission, New Delhi, India.

REFERENCES

1. Bachrach U, Brem S, Wertman S, Schnur LF and Greenblatt CL, *Leishmania spp:* cellular levels and synthesis of polyamines during growth cycles. *Exp Parasitol* **48**: 457–463, 1979.
2. WHO Expert Committee, *The Leishmaniasis*. World Health Organization, New York, 1984.
3. Berman JD, Experimental chemotherapy of leishmaniasis—a critical review. In: *Leishmaniasis* (Eds. Chang KP and Bray R), pp. 111–138. Elsevier Science Publishing, Inc., New York, 1985.
4. Black A, Induced cell differentiation in cancer therapy. *Cancer Treat Rep* **68**: 199–205, 1984.
5. Birt FD, Update on the effects of vitamins A, C and E and selenium on carcinogenesis. *Proc Soc Exp Biol Med* **183**: 311–320, 1986.
6. Ip C, Feasibility of using lower doses of chemopreventive agents in a combination regimen for cancer protection. *Cancer Lett* **39**: 239–246, 1988.
7. Hocman G, Chemotherapy of cancer: selenium. *Int J Biochem* **20**: 123–132, 1988.
8. Kozumbo WJ, Seed JL and Kensler TW, Inhibition by 2-(3)-tert-butyl-4-hydroxyanisole and antioxidants of epidermal ornithine decarboxylase activity induced by 12-*O*-tetra decanoyl phorbol-13-acetate. *Cancer Res* **43**: 2555–2559, 1983.
9. Eccles SA, Effects of retinoids in growth and dissemination of tumors; immunologic considerations. *Biochem Pharmacol* **34**: 1599–1610, 1985.
10. Lipmann SM, Kessler JF and Meyskens FL, Retinoids as preventive and therapeutic anticarcinogenic agents. *Cancer Treat Rep* **71**: 391–405, 1987.
11. Lotan R and Nicolson GL, Inhibitory effects of retinoic acid or retinol acetate on the growth of untransformed, transformed and tumor cells *in vitro*. *J Natl Cancer Inst* **59**: 1717–1722, 1977.
12. Medawar PB and Hant R, Anticancer action of retinoids. *Immunology* **42**: 349–353, 1981.
13. Medina D, Lane HW and Tracey CM, Selenium of mouse mammary tumorigenesis: an investigation of possible mechanisms. *Cancer Res* **43**: 2460–2464, 1983.
14. Patek PO, Collins JL, Yogeswaran G and Dannert G, Antitumor potential of retinoic acid: stimulation of immune mediated effectors. *Int J Cancer* **24**: 624–628, 1979.
15. Haddox ME and Russell DH, Cell cycle specific locus of vitamin A inhibition of growth. *Cancer Res* **39**: 2476–2480, 1979.
16. O'Brien JG, Simsiman RC and Boutwell RK, Induction of polyamine biosynthetic enzymes in mouse epidermis and their specificity for tumor promotion. *Cancer Res* **35**: 2426–2433, 1975.
17. Verrando P, Jullin L, Lacour JP, Ontonni JP, Immunohistochemical localization of ornithine decarboxylase in human skin. *J Dermatol* **14**: 112–117, 1987.
18. Bacchi CJ, Veraga C, Lipschik GY and Hunter SH, Synthesis and content of polyamines in bloodstream *Trypanosoma brucei*. *J Protozol* **26**: 484–488, 1979.
19. Schnur LF, Bachrach U, Greenblatt CL and Ben Joseph M, Polyamine synthesis and levels during the growth

and replication of *Leishmania toropica minor* and *Leishmania ethiopica*. *FEBS Lett* **106**: 202–206, 1979.
20. Mukherjee T, Ray M and Bhaduri A, Aspartate transcarbamylase from *Leishmania donovani*. *J Biol Chem* **263**: 708–713, 1988.
21. Kar K, Mukherji K, Naskar K, Bhattacharya A and Ghosh DK, *Leishmania donovani*: a chemically defined medium suitable for cultivation and cloning of promastigotes and transformation of amastigotes to promastigotes. *J Protozol* **37**: 277–279, 1990.
22. Seiler N, Use of classification in biochemical analysis. *Methods Biochem Anal* **16**: 289–337, 1970.
23. Seeley JE, Poso H and Pegg AE, Effect of androgens on turnover of ornithine decarboxylase in mouse kidney. *J Biol Chem* **257**: 7549–7553, 1982.
24. Athar M, Lloyd JR, Bickers DR and Mukhtar H, Malignant conversion of ultraviolet radiation and chemically reduced compounds. *Carcinogenesis* **10**: 1841–1845, 1989.
25. Athar M, Mukhtar H, Bickers DR, Khan IU and Kalyanaraman B, Evidence for the metabolism of tumor promotion organic hydroperoxides into free radicals by human carcinoma skin keratinocytes. An ESR-spin trapping study. *Carcinogenesis* **10**: 1499–1503, 1989.
26. Athar M, Raza H, Bickers DR and Mukhtar H, Inhibition of benzoyl peroxide mediated tumor promotion i.e. 7,12-dimethylbenz[*a*]anthracene-initiated skin of sencar mice by antioxidants nordihydroguaiaetic acid and diallyl sulfide. *J Invest Pharmacol* **94**: 162–165, 1990.
27. Black HS and Tiggs J, Evaluation of structurally related phenols for antiphotocarcinogenic and photoprotective properties. *Photochem Photobiol* **43**: 403–408, 1986.
28. Orille A, Levander, selenium and sulfur antioxidant protective systems, relationships with vitamin E and malaria. *Proc Soc Exp Biol Med* **200**: 255–259, 1992.
29. Taniquchi S, Kono T, Mizano N, Ishu M, Matsui-Yuasa I, Otani S and Hamada J, Effects of butylated hydroxyanisole on ornithine decarboxylase activity and its gene expression induced by phorbol ester tumor promotion. *J Invest Dermatol* **96**: 289–291, 1991.