*Biochemical Pharmacology*, Vol. 32, No. 22, pp. 3447–3451, 1983.
Printed in Great Britain.

0006–2952/83 $3.00 + 0.00


# BROMOACETYL-L-CARNITINE: BIOCHEMICAL AND ANTITRYPANOSOMAL ACTIONS AGAINST *TRYPANOSOMA BRUCEI BRUCEI*

ROBERT J. GILBERT and ROGER A. KLEIN
MRC Biochemical Parasitology Unit, Molteno Institute, University of Cambridge, Downing Street, Cambridge CB2 3EE, England
and
PALEY JOHNSON
Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, England

(*Received* 30 *April* 1983; *accepted* 24 *June* 1983)

**Abstract**—One of the causative agents of the African Trypanosamiases, *Trypanosoma brucei brucei* is able to use high intracellular carnitine concentrations and a high carnitine acetyl transferase (CAT) activity to stimulate ATP production. This paper reports that a carnitine analogue, bromoacetyl-L-carnitine, is an irreversible inhibitor of CAT from *T.brucei*, non-competitively inhibits carnitine uptake by *T.brucei* and has a potent *in vitro* effect against *T.brucei* motility and infectivity. An *in vivo* action in *T.brucei* infected mice is also reported. These results represent a new area of investigation in the important search for new antitrypanosomal agents.

*Trypanosoma brucei brucei* is a pathogenic, parasitic haemoflagellate protozoan of considerable veterinary and economic importance as one of the causative agents of the African trypanosoiases [1]; it is often used as a laboratory model of sleeping sickness in man and *Nagana* in cattle. The chemotherapy of these diseases is highly unsatisfactory: no new drug has been introduced for 25 years and those in current use are the subject of reports of widespread resistance [1, 2]. Without a functional tricarboxylic acid cycle [3], bloodstream forms of *T.brucei* are largely dependent on glycolysis for ATP production [4], which we have recently demonstrated to be stimulated by carnitine [5]. There exists in *T.brucei* an internal carnitine concentration of 1–5 mM [6], comparable with the highest values reported in biological systems [7, 8] and considerably higher than that present in rat plasma [9]. In addition it has been shown [6] both that *T.brucei* can take up carnitine against this concentration gradient and that high levels of the enzyme carnitine acetyl transferase (CAT) are present in these organisms.

In view of these observations, we have examined the biochemical and antitrypanosomal actions of bromoacetyl-L-carnitine (BAC), an irreversible inhibitor of CAT from pigeon breast muscle [10]. We report here that, in addition to the inhibition of carnitine-stimulated ATP synthesis [5], BAC, is an irreversible inhibitor of CAT from *T.brucei*, and is a non-competitive inhibitor of carnitine uptake in these organisms. BAC is also shown to have a potent action against *T.brucei* motility and infectivity *in vitro*, and a trypanocidal effect *in vivo* in *T.brucei*-infected mice.

## 1. MATERIALS AND METHODS

### 1.1 *Trypanosomes*

Rats (CFY strain) were used as laboratory hosts for *Trypanosoma brucei brucei* strain 427, which gives a monomorphic infection in rats [11]. Parasites were isolated as described previously [6]; blood was obtained by aortic puncture and trypanosomes were separated according to [12] as adapted from [13].

### 1.2 *Measurement of CAT activity*

Trypanosomes were lysed with 0.5% (w/v) Triton X-100 followed by centrifugation at 40,000 $g_{av}$ for 90 min. The supernate containing enzyme activity (acetyl CoA-L-(−)-carnitine-O-acetyl transferase; EC 2.3.1.7) was stored at −18° in 0.2 ml aliquots and assayed in a medium containing 100 mM Tris-HCl (pH 8.1, 21°) 0.5 mM 4-4′-dithobispyridine (DTBP), 0.1 mM acetyl CoA and carnitine and BAC as indicated. Release of free sulphydryl groups from the hydrolysis of acetyl CoA was calculated assuming an extinction coefficient for the pyridone of $E_{324} = 1.98 \times 10^4 \text{l} \cdot \text{mol}^{-2} \cdot \text{cm}^{-1}$ [14].

### 1.3 *Measurement of carnitine uptake of* T.brucei

Trypanosomes ($5 \times 10^8$ organisms per ml) were suspended in incubation buffer (IB) (4.99 mM KCl, 80.1 mM NaCl, 2.0 mM $MgCl_2$, 16.2 mM $Na_2HPO_4$, 3.8 mM $NaH_2PO_4$. 20 mM D-glucose, 1.5 g/l bovine serum albumin, pH 7.4) at 37° with radioactively labelled L-carnitine, prepared as described by Stokke and Bremer [15] and purified as described previously [6] to a specific activity of 5.3 k Bq/mole (0.14 μci/mol). Carnitine uptake was determined by centri-

fuging samples (0.1 ml) of the incubation mixture in microreaction tubes containing IB plus 0.5 mM unlabelled carnitine as stop medium, and a phthalate ester mixture as described previously [6, 16].

### 1.4. *Determination of the* in vitro *effects of BAC against trypanosome infectivity and motility*

Trypanosome motility was measured using photon correlation spectroscopy [17] which has the advantage over conventional light microscope techniques of being more objective, having greater sensitivity and being able to sample the three dimensional velocity distribution of $10^3$–$10^4$ cells in a very short time without the complication of wall effects. Mobility and apparent root mean square velocity were determined for trypanosomes resuspended in IB at 35° with BAC as indicated, using 5 and 20 degree scattering angles, a Malvern correlator and a He–Ne laser.

Trypanosome infectivity was determined following incubation of cells with BAC dissolved in IB at 37°. Groups of 5 mice were inoculated i.p. with 0.1 ml trypanosome suspension and tail blood was examined microscopically for the presence of parasites for 30 days after inoculation. Loss of infectivity was defined as being when trypanosomes were absent from tail blood for this period.

### 1.5. *Determination of the* in vivo *antitrypanosomal action of BAC in mice*

Mice, inoculated i.p. with $10^6$ *T.brucei*, were treated with a single 50 mg/kg i.p. dose of BAC at the times shown (inset to Fig. 3) and the time of death recorded. BAC was administered in 335 mM aqueous solution.

### 1.6. *Chemicals*

DTBP was obtained from the Aldrich Chemical Company and L-carnitine from the Sigma Chemical Company. BAC was synthesised as in [10]. All other chemicals were of the highest purity commercially available from major biochemical suppliers.

## 2. RESULTS

### 2.1. *CAT activity*

BAC shows the kinetics of an irreversible inhibitor of CAT from *T.brucei* (Fig. 1). The uninhibited reaction rate of 90.2 nanomoles free CoA released $min^{-1}$ (mg cell protein)$^{-1}$ (trace A) is greatly reduced by the presence of 70 μM or 350 μM BAC (traces B and C). This inhibitory effect was not reversed by the further addition of 6 mM L-carnitine (trace D) and was enhanced by preincubation of the CAT preparation with 70 μM BAC (trace E). We have also shown that incubation of whole trypanosomes *in vitro* with 70 μM BAC for 1 hr, followed by thorough washing prior to lysis, results in total inhibition of the CAT activity. It was not possible to confirm earlier findings, using pigeon breast muscle CAT [10], of increased inhibition by BAC in the presence of added CoA.

### 2.2. *Carnitine uptake*

The kinetics of inhibition of carnitine uptake by BAC are shown in Fig. 2. BAC is seen to be a non-competitive inhibitor of this process with an apparent $K_i$, determined graphically [18], of 351 ± 37 μM ($n = 48$) compared to the Km for carnitine

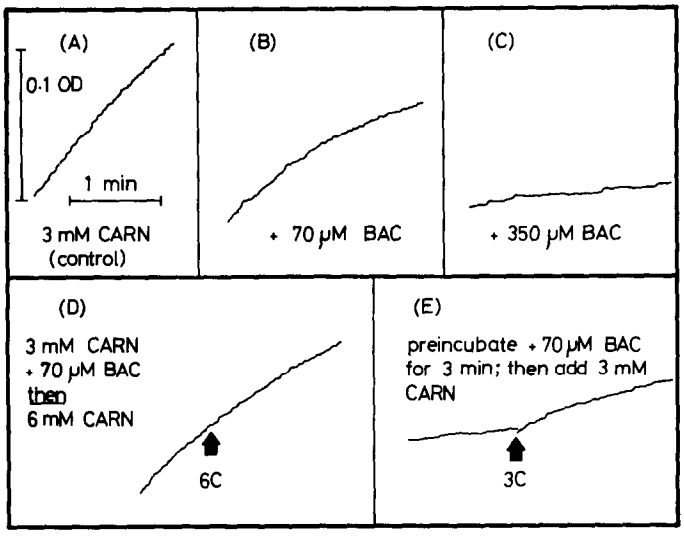


Fig. 1. The effect of BAC on the carnitine acetyl transferase activity isolated from *T.brucei*. CAT activity was measured in cell extracts prepared by trypanosome lysis with 0.5% (w/v) Triton X-100 followed by centrifugation at 40,000 $g_{av}$ for 90 min. The supernate was assayed for CAT activity in a medium containing 100 mM Tris-HCl (pH 8.1), 0.5 mM DTBP, 0.1 mM Acetyl CoA and L-carnitine (CARN) or bromoacetyl-L-carnitine (BAC) as indicated. Calculated rates of acetyl coenzyme A hydrolysis (nmoles free CoA released $min^{-1}$ [mg cell protein]$^{-1}$) ± S.E.M. ($n = 4$) are as follows: Trace A (control 90.2 ± 9.7, trace B (with 70 μM BAC) 44.6 ± 5.1, trace C (with 350 μM BAC) 6.68 ± 1.1, trace D (3 mM carnitine with 70 μM BAC, then 6 mM carnitine) 46.4 ± 5.1 and trace E (preincubation with 70 μM BAC, then with 3 mM carnitine) 24.5 ± 3.1.

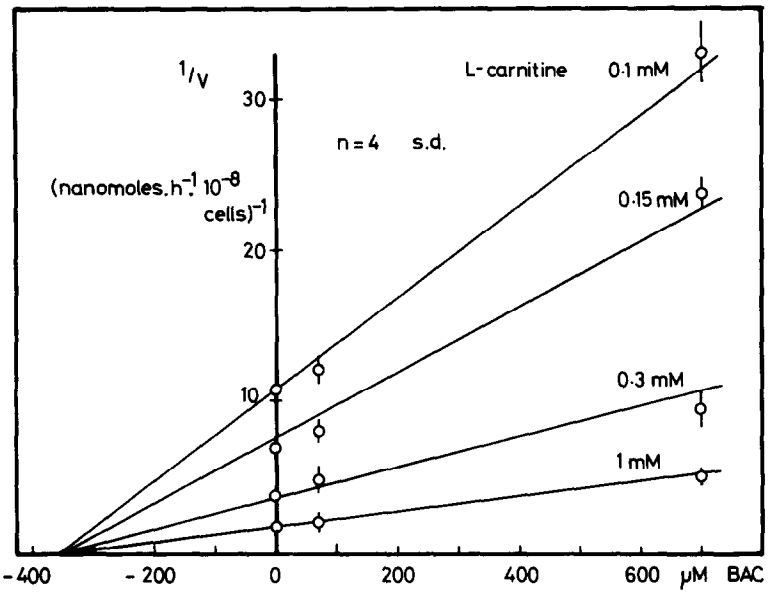


Fig. 2. Kinetics of inhibition of carnitine uptake by BAC in *T.brucei*. Shown is a Dixon plot [18] of (initial rate)$^{-1}$ plotted against inhibitor concentration with varying substrate concentrations. Incubation buffer (I.B.) containing $5 \times 10^8$ cells/ml and radioactively labelled L-carnitine was maintained at 37°. Uptake of L-carnitine was determined by centrifuging samples (0.1 ml) of the incubation mixture in microcreation tubes containing I.B. plus 0.5 mM unlabelled L-carnitine, and a phthalate ester mixture as described [6, 16].

at 37° of 572 ± 28 μM ($n$ = 24). Uptake was shown to be linear over 15 min in the absence of BAC.

### 2.3. *Effects of BAC on trypanosome infectivity and motility*

In view of the correlation between ATP levels and motility in other systems [19–21] we examined the effect of BAC on motility and infectivity of *T.brucei*. Following the addition of 50 μM BAC, the percentage of motile organisms (Fig. 3, curve (a)) dropped from 86% at the time of addition of BAC to 53% fifteen minutes later, and 28% after 45 min. A similar

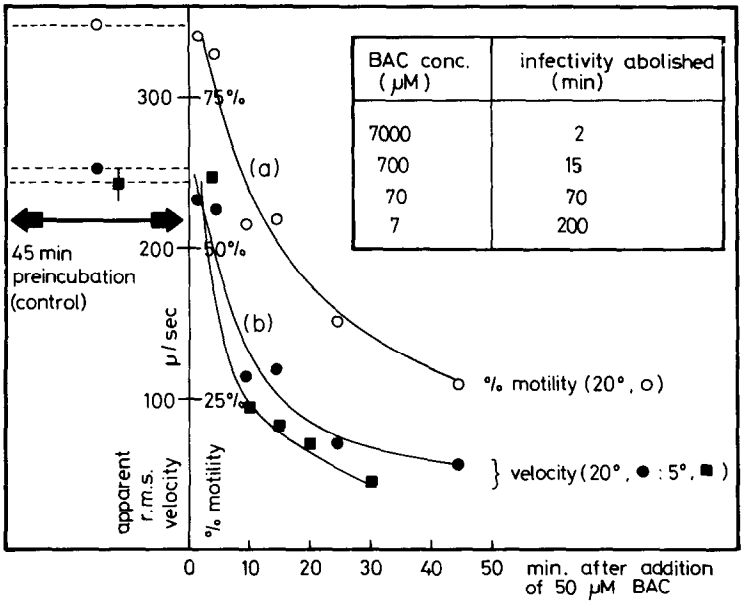


Fig. 3. The effect of BAC on the motility of *T.brucei in vitro*. Motility and apparent root mean square velocity were measured by photon correlation spectroscopy [17] (5 and 20 degree scattering; Malvern correlator; He–Ne laser). Cells were incubated in incubation buffer at a parasite density of $10^7$/ml at 35° and 50 μM BAC was added as shown. Infectivity was determined by inoculating groups of 5 mice i.p. with 0.1 ml of a suspension of trypanosomes treated with BAC at the concentrations shown above. Cells were incubated in incubation buffer at a parasite density of $10^8$/ml and at 37°.

decrease in the apparent root mean square velocity of the trypanosomes was also observed (Fig. 3, curve (b)). These results were obtained at a scattering angle of 20°; similar values for the r.m.s. velocity were obtained at a scattering angle of 5° and those are also shown in Fig. 3.

Parasite infectivity for mice decreased on incubation with increasing BAC concentration. Exposure to 700 μM BAC caused complete loss of infectivity after 15 min, while 7 μM BAC had a similar effect after 200 minutes (inset to Fig. 3). Untreated trypanosomes always killed mice in 3–6 days.

### 2.4. In vivo *trypanocidal effect of BAC*

Treatment of *T.brucei*-infected mice with a single i.p. dose of BAC (50 mg/kg) extended the life of those mice compared with untreated mice with similar infections; this dose was much less than the maximum tolerated dose since 150 mg/kg BAC was shown to have no obvious acute or chronic toxic effects on mice to which it was administered. Table 1 shows the effects of BAC administration at different times after inoculation of mice with *T.brucei*. Administration of BAC immediately after infection had the greatest antiparasitic effect, prolonging the life of these mice by 100%.

## 3. DISCUSSION

We have demonstrated three biochemical effects of BAC against *T.brucei in vitro*; inhibition of carnitine-stimulated ATP synthesis [5], irreversible inhibition of CAT activity and non-competitive inhibition of carnitine uptake by *T.brucei*. These effects are accompanied by loss of motility and infectivity following *in vitro* exposure to BAC and there is an *in vivo* trypanocidal action of BAC in *T.brucei* infected mice.

The irreversible action of BAC against CAT from pigeon breast muscle is known to be enhanced by the presence of added CoA [10]. That we were unable to demonstrate this effect is probably due to the presence of catalytic amounts of free CoA in the trypanosome extract rendering further addition without effect. This action of BAC against CAT is highly specific as the production of bromacetyl coenzyme A is dependent on BAC being cleaved by CAT [10] and is unlikely to occur by other mechanisms.

We have reported trypanosome velocities as apparent root mean square values because the movement of these organisms is a complex mixture of rotational and translational modes. The values obtained at the relatively low scattering angle of 5° are a fair representation of the overall translational component, but we are currently investigating the relative contribution of various modes in greater detail. It is of interest to correlate the effect of 50 μM BAC in reducing the percentage motility of trypanosomes from 86% to 28% in 50 min (Fig. 3), a time scale comparable with that taken for the abolition of infectivity by 70 μM BAC in a seventy minute incubation (inset to Fig. 3). The lowest concentrations of BAC that we have used which show activity against *T.brucei* motility (25 μM) and infectively (7 μM) are lower than those of the veterinary trypanocides in current use, Berenil, Suramin and Ethidium [22]. A similar comparison of the *in vivo* action of BAC with that of other trypanocides indicates that BAC has much reduced activity; Berenil, for example, has been shown to prolong indefinitely the life of *T. rhodesiense* infected mice [23]. A direct comparison however, is not possible because of the much larger inoculum used in previous tests. No morphological changes were apparent at this dose level as judged by light microscopy.

We have no data on the level of BAC reached in the blood of infected mice and are therefore unable to compare the BAC levels showing biochemical or antitrypanosomal activity in *T.brucei* with those present *in vivo*. We do, however, feel that the antitrypanosomal activity observed *in vivo* with *T.brucei* infected mice combined with the *in vitro* actions of this compound, opens up the possibility of a new area of investigation in the important search for new drugs against trypanosomiasis, leading to the synthesis of further carnitine analogues and the screening of these compounds for possible *in vivo* trypanocidal activity.

Table 1. The *in vivo* antitrypanosomal effect of BAC on *T.brucei* infections in mice

| Mouse group | Time of BAC dosage (hr) | Time of death (hr) |
|---|---|---|
| A | 0 | 132–150 |
| B | 24 | 100–120 |
| C | 48 | 90–100 |
| D | No drug (control) | 70–76 |

Each group comprised 10 mice, (2 × 5; done on separate occasions), which were infected with $10^6$ *T.brucei* i.p. at zero time, and then treated with a single i.p. dose of BAC (50 mg/kg) at the times shown.

*Acknowledgements*—We wish to thank Judith Angus for technical assistance. This work was partially supported by the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases.

## REFERENCES

1. WHO/FAO Technical Report series No. 635 (1979).
2. J. Williamson, *Trans. Roy. Soc. Trop. Med. Hyg.* **70**, 117 (1976).
3. I. W. Flynn and I. B. R. Bowman, *Comp. biochem. Physiol.* **45B**, 25 (1973).
4. P. T. Grant and J. R. Sargent, *Biochem. J.* **76**, 229 (1960).
5. R. J. Gilbert and R. A. Klein, *FEBS Lett.* **141**, 271 (1982).
6. R. A. Klein, J. M. Angus and A. E. Waterhouse, *Mol. biochem. Parasitol.* **6**, 93 (1982).
7. C. J. Rebouche, *Biochim. biophys. Acta* **471**, 145 (1977).
8. G. Cederblad and S. Linstedt, *Archs biochem. Biophys.* **175**, 173 (1976).
9. D. E. Brooks and J. E. A. McIntosh, *Biochem. J.* **148**, 439 (1975).
10. J. F. A. Chase and P. K. Tubbs, *Biochem. J.* **111**, 225 (1969).
11. G. A. M. Cross and J. C. Manning, *Parasitology* **67**, 315 (1973).

12. D. J. Linstead, R. A. Klein and M. V. Wheeler, *Parasitology* **71**, 93 (1975).
13. S. M. Lanham, *Nature* **218**, 1275 (1968).
14. D. R. Grassetti and J. F. Murray, *Archs biochem. Biophys.* **119**, 41 (1967).
15. O. Stokke and J. Bremer, *Biochim. biophys. Acta* **218**, 552 (1970).
16. J. Mowbray, *FEBS Lett.* **44**, 344 (1974).
17. R. E. Godfrey, P. Johnson and C. J. Stanley, in *Biomedical Applications of Light Scattering* (Eds. D. B. Sattelle, W. I. Lee and B. R. Ware), p. 373. Elsevier Biomedical Press, Amsterdam (1982).
18. M. Dixon and E. C. Webb, in *The Enzymes*, 2nd Edition, p. 325. Longmans, Green and Company Ltd., London (1964).
19. M. E. J. Holwill and J. Wais, *J. Exp. Biol.* **82**, 177 (1979).
20. M. E. J. Holwill and J. L. McGregor, *J. Exp. Biol.* **65**, 229 (1976).
21. B. H. Gibbons and I. R. Gibbons, *J. Cell. Biol.* **63**, 785 (1974).
22. J. Williamson and T. J. Scott-Finnegan, *Antimicrob. ag. Chemotherap.* **13**, 735 (1978).
23. L. Rane, D. S. Rane and K. E. Kinnamon, *Am. J. Trop. Med. Hyg.* **25**, 395 (1976).