Biochemical Pharmacology, Vol. 38, No. 23, pp. 4327–4332, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

EFFECTS OF INTERMEDIARY METABOLITES AND ELECTRON TRANSPORT INHIBITORS ON ACTION OF CHLOROQUINE ON *BRUGIA PAHANGI* AND *ONCHOCERCA VOLVULUS*

ELIZABETH A. VANDEWAA,*† JEFFREY F. WILLIAMS‡ and TIMOTHY G. GEARY§

*Department of Pharmacology and Toxicology, and ‡Department of Microbiology and Public Health, Michigan State University, East Lansing, MI 48824; and §Parasitology Research, The Upjohn Company, Kalamazoo, MI 49001, U.S.A.

(*Received* 18 *July* 1988; *accepted* 24 *May* 1989)

Abstract—We examined the possibility that chloroquine is interfering with aerobic energy-generating processes in the adult filarial parasites, *Brugia pahangi* and *Onchocerca volvulus*. Using motility of these parasites as an assay of drug effect, we found that micromolar concentrations of chloroquine caused significant paralysis, but only in alkaline medium (pH 8.4). The addition of 12 mM glutamine or 10 mM albizziin to the medium completely antagonized drug-induced paralysis. In addition, in *B. pahangi*, all of the tricarboxylic acid cycle intermediates (10 mM) except citrate and pyruvate antagonized the effect of chloroquine on motility; in *O. volvulus*, oxaloacetate as well as glutamine inhibited the effect of the drug. The effect of chloroquine on both parasites was enhanced when it was used in combination with 10 μM acivicin, a glutamine antimetabolite. Here motility of *B. pahangi* was reduced significantly within 24–48 hr at acidic (6.8) neutral (7.4) and alkaline (8.4) pH. This effect was partially reversible by glutamine (12 mM). Motility of *O. volvulus* was reduced to near zero within 4 hr with this drug combination. Antimycin A and rotenone, both electron transport inhibitors, also synergized with chloroquine at any pH to produce paralysis in *B. pahangi*. The effects of the rotenone and chloroquine combination were reversed in the presence of 10 mM succinate. However, glutamine (12 mM) was unable to antagonize the effects of chloroquine plus antimycin A on the motility of *B. pahangi*. These findings suggest that chloroquine may be inhibiting aerobic energy metabolism in the filariae, possibly at the level of electron transport. Furthermore, since chloroquine is well-tolerated but only weakly filaricidal *in vivo*, the data indicate that use of this drug in combination with other inhibitors of aerobic energy metabolism may be a chemotherapeutically useful approach to the treatment of filariases.

Few drugs are currently available for the treatment of human filariases. In recent work, we and others have shown that several species of filariae are sensitive to quinoline-containing antimalarials such as chloroquine (CQ) *in vitro* [1, 2]. This drug and others in its class also have some activity *in vivo* against *Wuchereria bancrofti* [3] and microfilariae of *Onchocerca volvulus* [4]. However, the drug is not sufficiently efficacious to warrant its use in the treatment of human filariases.

The mechanism of action of the aminoquinolines against the filarial parasites is not well understood. We have shown in earlier work that CQ causes a dose-dependent decrease in lactate production in *Brugia pahangi* [1]. With respect to other parasites, it has been found that CQ inhibits the uptake of glucose by *Schistosoma mansoni* [5], and in *Ascaris suum*, CQ inhibits electron transport processes [6]. It has been suggested that CQ may act in malaria parasites by interfering with processes of aerobic energy generation [7]. Energy metabolism is becoming increasingly understood in the filariae [8, 9], so it is reasonable to ask whether or not CQ is interfering with these processes in the filarial parasites.

In the present study, we investigated the ability of energy substrates to antagonize the effects of CQ on *B. pahangi* and *O. volvulus* adults *in vitro*. In addition, studies in which CQ was combined with compounds known to interfere with energy metabolism were undertaken in order to better understand the mechanism of action of this drug in filariae.

† Correspondence: Dr Elizabeth A. VandeWaa, Department of Comparative Biosciences, University of Wisconsin, 2015 Linden Drive West, Madison, WI 53706.

MATERIALS AND METHODS

Parasites. Adult *B. pahangi* were removed from the peritoneal cavity of infected male mongolian jirds. Female parasites were separated from males and were placed in sterile medium consisting of RPMI 1640 [buffered with 20 mM *N*-α-hydroxyethylpiperazine-*N'*-α-ethanesulfonic acid (Hepes), pH 7.4] plus 100 units/ml penicillin, 0.1 mg/ml streptomycin and 0.24 μg/ml amphotericin B (all medium components from Gibco). *O. volvulus* adult females were digested free from nodules using the method of Schulz-Key and coworkers [10]. Briefly, nodules were surgically removed from patients in Zapallo Grande, Ecuador, and removal of parasites from nodule tissue was accomplished using collagenase. Typically worms were freed from the nodules within 20 hr or less. Female *O. volvulus* were used only if they were actively moving and had sustained no apparent damage upon removal from the host or during digestion. After isolation, worms were rinsed

several times in the medium described above prior to assay.

Assay. Parasites were incubated at 37° in RPMI 1640 at pH 6.8, 7.4 or 8.4 as indicated. The pH of the media was adjusted following the addition of inhibitors or substrate, prior to the incubation of parasites. In all cases, a sufficiently large volume of medium was used so that the pH did not shift more than 0.3 units throughout the incubation. At certain times during incubation, parasite motility was measured to assess drug effects. Motility has been shown previously to be a sensitive indicator of drug effects on filarial parasites [11, 12], and was particularly useful in these experiments since *O. volvulus* parasites were limited. To measure motility of *O. volvulus*, worms were viewed in culture using an inverted microscope at regular intervals pre- and post-exposure to compounds. The effects of drugs were quantified using a scoring system (0 to ++++); at least three control and three treated parasites were measured for each drug tested, and assays were performed in triplicate. Motility of *B. pahangi* was measured quantitatively using the motility meter as previously described [13]. For these parasites, four control worms were compared to four worms per treatment group, and the assays were carried out in triplicate. In certain experiments, motility of parasites exposed to drugs was expressed as percent of control worm motility (i.e. parasites were incubated in the same medium, but no drug or additional substrates were added). CQ was purchased from Boehringer-Mannheim (Indianapolis, IN). Acivicin was obtained from The Upjohn Co. (Kalamazoo, MI). Rotenone was purchased from the Aldrich Chemical Co. (Milwaukee, WI). All other inhibitors and intermediates were obtained from the Sigma Chemical Co. (St Louis, MO).

RESULTS

Reversal of the effect of CQ by glutamine, albizziin and tricarboxylic acid cycle intermediates. Figure 1a shows the effect of 10 μM CQ on the motility of *B. pahangi* incubated in RPMI 1640 at pH 6.8, 7.4 or 8.4. Parasites were almost completely paralyzed by CQ in alkaline medium by 24 hr. Glutamine (12 mM) in the culture medium completely antagonized the effects of CQ on parasite motility at pH 8.4 (Fig. 1b). Albizziin (10 mM), a structural analog of glutamine and a substrate for the enzyme which converts glutamine ultimately to α-ketoglutarate, similarly antagonized CQ at pH 8.4 (Fig. 1b).

The amount of glutamine necessary to antagonize CQ was titrated as shown in Fig. 2. Glutamine at 6 mM was able to protect parasites from CQ for up to 24 hr, but 12 mM was needed to completely antagonize the drug effect for the duration of the incubation.

Several tricarboxylic acid cycle intermediates also antagonized the effects of 10 μM CQ on the parasite at 72 hr. Malate and oxaloacetate (10 mM), like glutamine, were able to completely maintain parasite motility in the presence of CQ (Table 1). Isocitrate, succinate, fumarate and α-ketoglutarate were all able to support motility at levels of at least 100% of control for 72 hr in the presence of CQ. However,

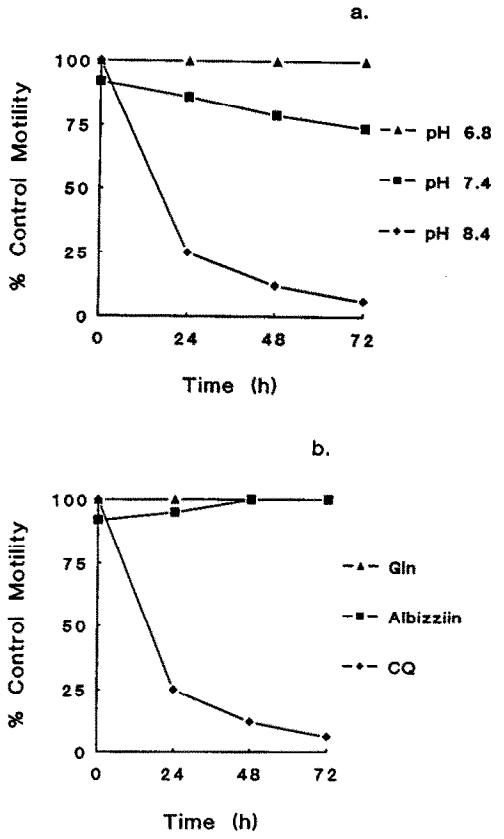

Fig. 1. (a) Effect of 10 μM chloroquine (CQ) on motility of *B. pahangi* in RPMI 1640 at pH 6.8, 7.4 and 8.4. Parasites were incubated in CQ, and motility recorded at 0, 24, 48 and 72 hr was compared to motility of parasites incubated in the absence of CQ. (b) Effect of 10 μM CQ, 10 μM CQ plus 12 mM glutamine, and 10 μM CQ plus 10 mM albizziin at pH 8.4. Parasites were incubated, and motility was recorded as described. Each point on panels a and b represents mean parasite motility of four worms per group in three separate experiments.

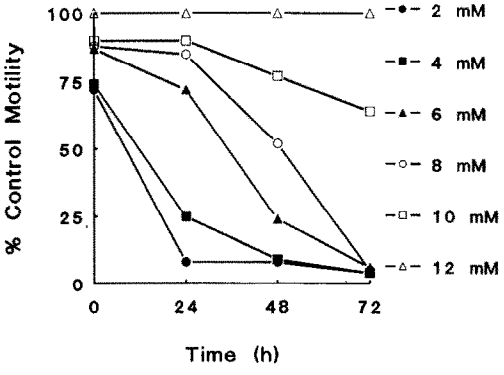

Fig. 2. Effect of glutamine on motility of *B. pahangi* exposed to 10 μM CQ. Parasites were incubated in RPMI 1640, pH 8.4, with 10 μM CQ and glutamine concentrations ranging from 2 to 12 mM. Each point represents the mean motility of three worms per group in four separate experiments.

Table 1. Effects of various substrates (10 mM) on motility of *B. pahangi* incubated in the presence or absence of chloroquine for 72 hr

Substrate	Motility index*	
	No CQ	10 μM CQ
None (RPMI 1640)	247.3 ± 34.5	19.5 ± 3.0
Glutamine	385.3 ± 67.0	379.0 ± 42.3†
α-Ketoglutarate	302.0 ± 38.7	248.8 ± 22.3†
Succinate	312.0 ± 32.1	252.3 ± 71.0†
Fumarate	366.0 ± 49.0	318.5 ± 20.6†
Malate	306.3 ± 40.5	391.0 ± 65.4†
Oxaloacetate	335.5 ± 80.8	408.9 ± 36.9†
Citrate	45.8 ± 25.2‡	26.5 ± 5.5
Isocitrate	349.3 ± 62.0	400.5 ± 74.8†
Pyruvate	318.3 ± 79.1	14.7 ± 1.6

* Data are the means ± SD of triplicate incubations. Parasites were incubated in RPMI 1640 (pH 8.4) with a 10 mM concentration of each substrate, ±10 μM CQ.

† Significantly different ($P < 0.05$) from motility of parasites incubated in RPMI 1640 plus 10 μM CQ.

‡ Significantly different ($P < 0.05$) from motility of parasites incubated in RPMI 1640 alone.

Table 2. Effects of inhibitors and substrates on motility of *O. volvulus*

Treatment	Motility index/Time* (hr)			
	0	6	12	24
Control	++++	++++	++++	++++
CQ	++++	+++	0	0
CQ/Gln	++++	++++	++++	++++
CQ/OAA	++++	++++	+++	+
Acivicin	++++	++++	+++	+
CQ/Acivicin	++++	0	0	0

* Data are mean values of triplicate incubations. Worms were incubated in RPMI 1640 (pH 8.4) alone or in RPMI 1640 containing 10 μM CQ or 10 μM acivicin, supplemented with 10 mM glutamine or 10 mM oxaloacetate (OAA).

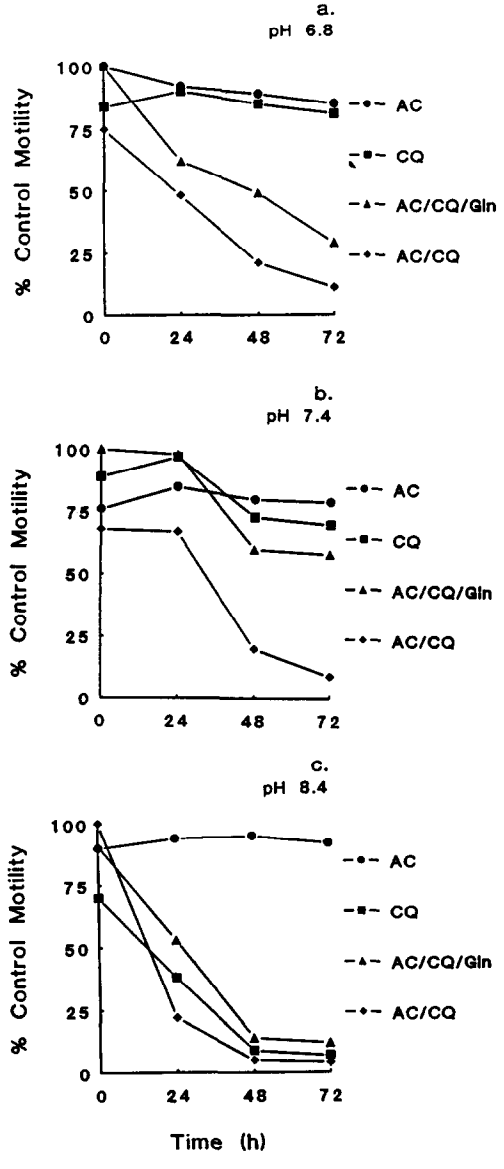

Fig. 3. Effects of acivicin (AC) and CQ on motility of *B. pahangi*. Parasites (N = 6) were incubated in RPMI 1640 at pH 6.8, 7.4 or 8.4 in the presence of 10 μM CQ, 10 μM AC, both AC and CQ, or both drugs plus 12 mM glutamine. Motility was recorded at 0, 24, 48 and 72 hr, and each point represents mean parasite motility at each time.

citrate and pyruvate at concentrations up to 12 mM were able to antagonize the effects of CQ for only 24 hr or less (Table 1).

O. volvulus females were also very sensitive to CQ at pH 8.4. Again, 12 mM glutamine antagonized this effect, whereas oxaloacetate (10 mM) was much less effective at antagonizing the effect of CQ on this parasite (Table 2).

Effect of drug combinations on parasite motility. Acivicin, a glutamine antimetabolite, had little effect on the motility of *B. pahangi* at 10 μM at pH 6.8, 7.4 or 8.4 over a 72-hr incubation period (Fig. 3). When this agent was combined with 10 μM CQ, however, significant paralysis ($P < 0.05$) was seen within 24 hr in medium at any pH tested (Fig. 3). At pH 6.8 and 7.4, the combination of drugs reduced motility by 80% in 48 hr. In alkaline medium, the effects of the combination were difficult to separate from the effects of CQ alone.

In *O. volvulus* females, 10 μM acivicin reduced parasite motility by approximately 75% in 24 hr (Table 2). When 10 μM CQ was combined with acivicin in the medium, parasites were completely immotile within 6 hr.

When CQ (10 μM) was combined with 1 μM antimycin A, an electron transport inhibitor, a synergistic effect was seen at pH 6.8 and 7.4. Antimycin A alone reduced motility of *B. pahangi* significantly (by 56%) by 72 hr in acidic medium, but the addition of CQ to this drug caused motility to decrease to 36% of control levels within 48 hr (Fig. 4a). At pH 7.4, antimycin A alone had no effect on motility,

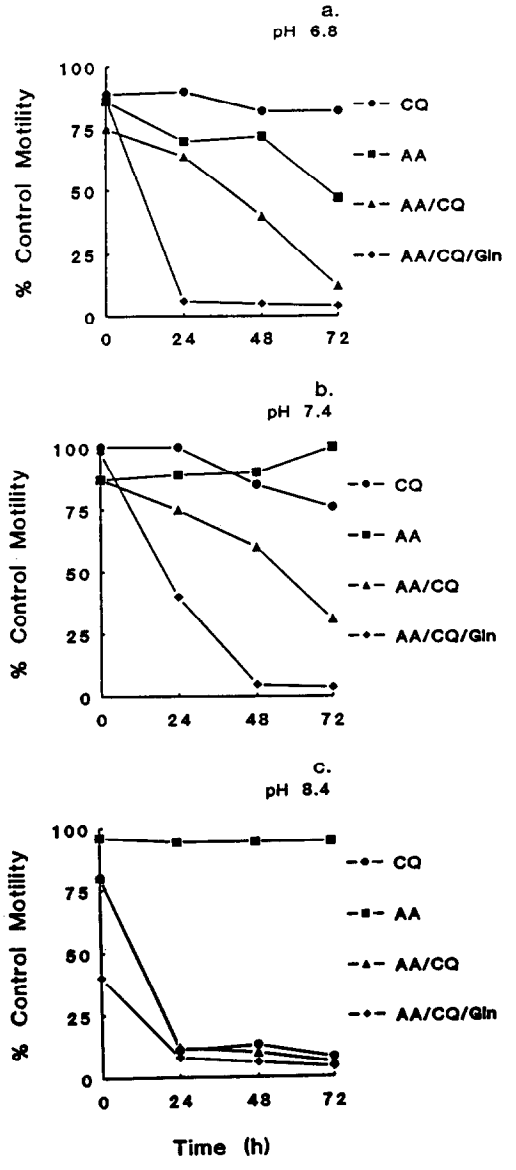

Fig. 4. Effects of antimycin A (AA) and CQ on motility of *B. pahangi* parasites were incubated as described in the legend of Fig. 3.

but in combination with CQ a 66% reduction was seen at 72 hr (Fig. 4b). Again, at pH 8.4, it was difficult to differentiate between effects of 10 μM CQ alone versus the combination of drugs (Fig. 4c). We tested 1 μM CQ in combination with 1 μM antimycin A and saw a 58% reduction in motility at pH 8.4 by 48 hr. CQ alone at this concentration caused a similar effect on motility by 72 hr (data not shown).

We attempted to antagonize the effects of the combination of drugs with glutamine. With the CQ and acivicin combination, the addition of 12 mM glutamine to the culture medium was able to partially antagonize the drug effects at pH 6.8 and 7.4 (Fig. 3, a and b) on *B. pahangi*. When antimycin A was used in combination with CQ (10 μM), however, the addition of glutamine did not restore motility in this parasite (Fig. 4). Succinate (10 mM) was also unable to reverse the effects of antimycin A and CQ (data not shown).

Rotenone, an inhibitor of NADH dehydrogenase activity, was also examined for its effects in the presence of CQ. At 0.1 μM, rotenone combined with 10 μM CQ caused a significant (>50%) decrease in motility of *B. pahangi* by 48 hr at pH 6.8 and 7.4, and within 24 hr at pH 8.4. Here, the addition of 10 mM succinate or 12 mM glutamine to the medium was able to completely reverse the effects of the drugs at any pH (data not shown).

DISCUSSION

The mechanism of action of the aminoquinolines in filarial parasites is not known. It appears that there may be a shift in parasite physiology in alkaline medium which renders the worms more susceptible to CQ, and this change may be reflected in the energy metabolism of the parasite under these conditions. The antagonism of CQ by tricarboxylic acid cycle intermediates suggests not only that these parasites can readily assimilate these substrates as sources of energy, but also that CQ may act as sites important for energy production. Finally, the synergism between CQ and other agents which disrupt aerobic energy metabolism supports this hypothesis and suggests that combination therapy may be effective in the treatment of filariasis.

In earlier work, we showed that increased sensitivity to the aminoquinolines at alkaline pH could not be explained by enhanced uptake of the drug under these conditions [14]. Thus, parasite physiological processes must be altered with changes in external pH. Indeed, we showed that, as the pH of the medium changed, a concomitant shift in parasite internal pH occurred. Some of the enzymes involved in energy-generating processes in nematodes show pH-dependent responses to anthelmintics [6]. Thus, the shift in pH may change the sensitivity of these organisms to CQ.

In both *B. pahangi* and *O. volvulus*, glutamine and oxaloacetate (as well as other tricarboxylic acid cycle intermediates in the case of *B. pahangi*) were able to antagonize CQ, suggesting that the parasites could utilize these substrates to avoid the effects of the drug. In vertebrates, one of the ways glutamine can be assimilated into the tricarboxylic acid cycle is via conversion to α-ketoglutaramate and then α-ketoglutarate by the enzyme phenylpyruvate glutamine aminotransferase (glutaminase II, EC 2.6.1.15) [15]. Albizziin, which is a structural analog of glutamine and can be similarly converted to α-ketoglutarate by the same enzyme, also reversed the effect of CQ, again suggesting a pathway in these parasites for the utilization of glutamine as an energy source. Citrate, a known inhibitor of the tricarboxylic acid cycle, did not reverse the effect of CQ. Pyruvate was less able to antagonize the effect of CQ than were other tricarboxylic acid cycle intermediates. While *B. pahangi* have measurable pyruvate dehydrogenase, its activity is extremely low relative to other tricarboxylic acid cycle enzymes [16], and this could account for the lack of effect of this intermediate.

When combined with acivicin, a glutamine antagonist, the efficacy of CQ increased at each pH. Acivicin interferes with cellular processes which rely on glutamine by acting as a competitive inhibitor of enzymes necessary for glutamine utilization [17]. The resulting depletion of glutamine in cells contributes to the antitumor effects of acivicin [18]. Chloroquine plus acivicin was a particularly efficacious combination against *O. volvulus*, completely inhibiting motility within 4 hr. CQ alone was most effective in alkaline medium, but the combination of acivicin and CQ caused marked paralysis at any pH. Thus, the combination may be effective under physiological conditions. Since few drugs are available for the treatment of river blindness, the disease caused by this organism, combination therapy might be a rational approach.

The combination of CQ and antimycin A was also effective in reducing motility of *B. pahangi*. Antimycin A disrupts electron transport by blocking the reduction of cytochromes c, c_1 and $a + a_3$. Since acivicin works to block the uptake of glutamine so that it cannot be utilized in aerobic pathways, while antimycin A acts to inhibit the respiratory electron transport chain, and both have synergistic effects with CQ, CQ itself may interact with pathways of aerobic energy generation in these parasites. Kohler and Bachmann [61] have shown that CQ (3 μM) inhibits the NADH dehydrogenase activity at the site at which the enzyme reduces the quinone in *Ascaris suum*, and propose that this action may contribute to nematocidal effects of CQ. That "rescue" of parasites from CQ by tricarboxylic acid cycle intermediates occurs, as well as synergism between CQ and both acivicin and antimycin A, lends further support to the hypothesis that CQ may be affecting aerobic energy-generating pathways in the filariae.

The paralysis induced by the combination of CQ and acivicin was partially reversed by 12 mM glutamine, suggesting that acivicin may be a competitive inhibitor of glutamine-using processes in the filariae. In tumor cells, glutamine also protects the cell from acivicin in a concentration-dependent manner [19]. On the other hand, glutamine was unable to protect parasites from the combination of CQ and antimycin A. This lack of effect is explicable if CQ inhibits the quinone reducing site of the mitochondrial NADH dehydrogenase as proposed [6]. Since antimycin A inhibits the reduction of cytochromes c, c_1 and $a + a_3$, the resulting sequential blocks in the electron transport chain would be irreversible by glutamine, or presumably, any tricarboxylic acid cycle intermediate.

In studies by Mendis and Townson [8], inhibition of oxygen consumption by rotenone (2.5 μM) in *B. pahangi* was reversed by succinate. We found an inhibition of motility when 0.1 μM rotenone and 10 μM CQ were used in combination. This inhibition was reversed by succinate. Rotenone inhibits the NADH dehydrogenase [8] as CQ is thought to; therefore, rescue of parasites exposed to CQ by tricarboxylic acid cycle intermediates could be due to the conversion of these substrates to succinate. The succinate formed, as in mammalian cells, could provide an alternate substrate input site to electron transport. *B. pahangi* and some *Onchocerca* species possess all the enzymes for the tricarboxylic acid cycle [9, 16], so metabolism of glutamine and other substrates to succinate via a full or partial tricarboxylic acid cycle operating in either a forward or reverse direction is feasible.

Our studies indicate that CQ may disrupt pathways for aerobic energy generation in both *B. pahangi* and *O. volvulus*. Whether or not this action of CQ is relevant *in vivo* is not known. If the inhibition of NADH dehydrogenase does contribute significantly to the filaricidal effect of CQ, its efficacy (or lack thereof) may indicate to what extent different filarial species rely on aerobic energy metabolism. CQ is a clinically useful antimalarial and antiarthritic drug which is well-tolerated by humans. However, if parasites had an efficient pathway which could utilize exogenous substrates to generate succinate and bypass the effect of CQ, less toxic derivatives of antimycin A used in combination with CQ might be chemotherapeutically useful. Additionally, acivicin has been given to humans as an antitumor agent, and concentrations which affected parasites *in vitro* in our studies were approximately 1000-fold less than amounts needed to affect tumor cells *in vitro* [19]. While the efficacy of CQ against adult filariae *in vivo* is variable, our data suggest that it could be enhanced and possibly extended to other species if used in combination with other agents.

Acknowledgements—We are grateful to Dr Ronald Guderian, Hospital Voz Andes, Ecuador, for providing nodules infected with *O. volvulus*. This investigation received support from the World Health Organization Onchocerciasis Chemotherapy Project of the Onchocerciasis Control Program in West Africa (Research Proposal No. 84011). E. A. V. is supported by NIH Training Grant AI 07203.

REFERENCES

1. VandeWaa EA, Bennett JL, Williams JF, Satti MZ and Geary TG, Effects of aminoquinolines on adult filarial parasites *in vitro*. *J Parasitol* **75**: 367–372, 1989.
2. Walter RD, Wittich RM and Kuhlow F, Filaricidal effect of mefloquine on adults and microfilariae of *Brugia patei* and *Brugia malayi*. *Trop Med Parasitol* **38**: 55–56, 1987.
3. McMahon JE, Preliminary screening of artifilarial activity of levamisole and amodiaquine on *Wuchereria bancrofti*. *Ann Trop Med Parasitol* **73**: 465–472, 1979.
4. Guderian RH, Williams JF and Mackenzie CD, Chloroquine and onchocerciasis. *Lancet* **84**: 807–808, 1986.
5. Magzoub M, Effects of chloroquine sulphate on glycolysis and motility of adult *Schistosoma mansoni* worms. *Sudan Med J* **10**: 145–148, 1972.
6. Kohler P and Bachmann R, The effects of the antiparasitic drugs levamisole, thiabendazole, praziquantel and chloroquine on mitochondrial electron transport in muscle tissue from *Ascaris suum*. *Mol Pharmacol* **14**: 155–163, 1978.
7. Howells RE, Peters W, Homewood CA and Warhurst DC, Theory for the mechanism of chloroquine resistance in rodent malaria. *Nature* **228**: 625–628, 1970.
8. Mendis AHW and Townson S, Evidence for the occurrence of respiratory electron transport in adult *Brugia pahangi* and *Dipetalonema viteae*. *Mol Biochem Parasitol* **14**: 337–354, 1985.
9. Dunn TS, Raines PS, Barrett J and Butterworth PE, Carbohydrate metabolism in *Onchocerca gutturosa* and *Onchocerca lienalis* (Nematoda: Filaroidea). *Int J Parasitol* **18**: 21–26, 1988.

10. Schulz-Key H, Albiez EJ and Buttner DW, Isolation of living adult *Onchocerca volvulus* from nodules. *Trop Med Parasitol* **28**: 428–430, 1977.
11. Court JP, Martin-Short M and Lees GM, A comparison of the response of *Dipetalonema viteae* and *Brugia pahangi* adult worms to antifilarial agents *in vitro*. *Trop Med Parasitol* **37**: 375–380, 1986.
12. Nowak M, Hutchinson GW and Copeman DB, *In vitro* drug screening in isolated male *Onchocerca gibsoni* using motility suppression. *Trop Med Parasitol* **38**: 128–130, 1987.
13. Bennett JL and Pax RA, Micromotility meter: an instrument designed to evaluate the action of drugs on motility of larval and adult nematodes. *Parasitology* **93**: 341–346, 1986.
14. VandeWaa EA, Williams JF and Geary TG, pH-Dependent uptake and macrofilaricidal effects of chloroquine on adult filarial parasites *in vitro*. *Exp Parasitol* **68**: 31–39, 1989.
15. Kupchik HZ and Knox WE, Assays of glutamine and its aminotransferase with the enol-borate of phenylpyruvate. *Arch Biochem Biophys* **136**: 178–186, 1970.
16. Barrett J, Mendis AHW and Butterworth PE, Carbohydrate metabolism in *Brugia pahangi* (Nematoda: Filarioidea. *Int J Parasitol* **16**: 465–469, 1986.
17. Prajda N, Enzyme targets of antiglutamine agents in cancer chemotherapy. *Adv Enzymes Regul* **24**: 207–223, 1985.
18. Weber B, Lui MS, Sebolt J and Faderan MA, Molecular targets of antiglutamine therapy with acivicin in cancer cells. In: *Glutamine Metabolism in Mammalian Tissues* (Eds. Haussinger D and Sies H), pp. 1–56. Springer, New York, 1984.
19. Niel GL, Berger AE, McPartland RP, Grindey GB and Bloch A, Biochemical and pharmacological effects of the fermentation-derived antitumor agent ($\alpha S,5S$)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid (AT-125). *Cancer Res* **39**: 852–856, 1979.