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Inhibitors of choline transport into *Plasmodium*-infected erythrocytes are effective antiplasmodial compounds *in vitro*

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The increasing resurgence of *Plasmodium falciparum* malaria, linked to the resistance both of mosquitoes and parasites to various pesticides and conventional drugs, calls for new therapeutic approaches to this endemic disease [1]. Interfering with parasite-specific metabolic pathways could lead to a new range of antimalarial drugs whose structure and mode of action are different from those currently used.

Phospholipid (PL) metabolism was chosen as a target because of its magnitude and specificity. Although little or no enzymatic machinery devoted to PL biosynthesis exists in mature mammalian erythrocytes [2–6], an increase amounting to as much as 500% of the host erythrocyte PL content occurs after infection. New molecules of PL are manufactured by the parasite itself, from precursors such as fatty acids and polar heads constantly drawn from the plasma [6]. Any compounds that can curtail the availability of these precursors should be deleterious to the parasite.

We have previously shown that D-2-amino-1-butanol which is incorporated as a polar head into unnatural PL, has a deleterious effect on *Plasmodium* growth, suggesting that PL metabolism is essential to the parasite [7]. Since phosphatidylcholine (PC) is the most abundant PL in infected erythrocytes [2], in the present work, we tested compounds likely to interfere with choline incorporation. Based on their analogy with choline and on previous results obtained in other cellular systems, such as erythrocytes and the nervous system [8–10], it was probable that quaternary-ammonium-containing compounds would affect choline utilization by infected erythrocytes, and consequently block parasite development *in vitro*.

Materials and methods

Bromide salts of decyltrimethylammonium, decamethonium and hemicholinium 3 were from Sigma, St. Louis, MO, U.S.A. Modified RPMI 1640 containing 40 μM choline and inositol, and 140 μM serine, was provided by Eurobio, France.

The Nigerian strain of *P. falciparum* (Dr W. H. Richard, Wellcome Research Laboratory, Beckenham, U.K. [11]) was maintained in continuous culture according to the Petri-dish candle-jar method [12].

Splenectomized *Macaca fascicularis* monkeys (Sanofi, Montpellier, France), were infected by cryopreserved [13] *P. knowlesi* (Washington strain, variant 1, from Dr G. Mitchell, Guy's Hospital, London, U.K.), and infected erythrocytes were supplied as described previously [7].

Biochemical studies. P. knowlesi-infected erythrocytes were preincubated for various times at 37° in the presence of the different drugs and in medium composed of modified RPMI 1640 with 25 mM Hepes (pH 7.4). They were then incubated with radioactive precursors in the same medium enriched with 1 mM ATP, 30 μM CoA and 0.5 mM of the essential plasmatic unesterified fatty acids, i.e. palmitic, stearic, oleic and linoleic acids in a molar ratio of 1.7/0.7/1.3/1.3, respectively, bound to fat-free bovine serum albumin (17–20 mg/ml). Reactions were stopped at +4°. The cells were then pelleted at 10^4 g/min and washed at 4°, once with 4 ml modified RPMI and twice with 4 ml 0.9% NaCl containing 40 μM cold choline. Cellular lipids were extracted according to the method of Folch *et al.* [14] as modified [15], and then fractionated as described previously [7]. The aqueous extracts of the Folch procedure were harvested and evaporated at 37° under an N_2 stream. Residues were resuspended in ethanol–water (1–1); choline and its metabolites were then separated by silica gel TLC [16]. After visualization of appropriate standards with iodine vapor, radioactive spots were counted with a Packard 460 CD liquid scintillation spectrometer.

When [^{3}H(G)]hypoxanthine (NEN) or [U-^{14}C]isoleucine (Amersham International, Amersham, U.K.) incorporation into macromolecules was monitored, 4 ml of 10% cold trichloroacetic acid (TCA) was added to washed cells. The precipitates were washed twice with 4 ml 10% TCA, then solubilized in 0.8 ml NCS (Amersham) and counted for radioactivity.

Results and discussion

The present results demonstrate that analogs of choline, containing one or two quaternary ammoniums, i.e. decyltrimethylammonium (DTMA), or decamethonium (DM) and hemicholinium 3 (HC3), are lethal to *Plasmodium falciparum in vitro* in a dose-dependant manner, with very

low IC_{50} (concentration resulting in 50% inhibition growth) of 7×10^{-7}, 10^{-6} and 4×10^{-6} M, respectively. Parasitemia, expressed as % of control, vs drug concentration described a sigmoid curve: the antimalarial effects generally occurred over a narrow range of concentrations between 4×10^{-7} M and 4×10^{-6} M. Hemicholinium 3 was the only compound exerting its effect over a wider range, with 10% inhibition at 4×10^{-7} M and 100% at 4×10^{-5} M (Fig. 1).

It should be noted that the critical micellar concentrations of these compounds are at least three orders of magnitude higher than the IC_{50} observed for antiplasmodial activity [17]. Moreover, we checked that under our conditions, their haemolytic concentration 50 (concentration resulting in 50% erythrocyte lysis), measured after 48 hr contact between erythrocytes and DM, HC3, or DTMA, were higher than 7×10^{-3}, 4×10^{-3} and 10^{-3} M, respectively, i.e. again more than 1000 times their IC_{50}. Hence, the antiplasmodial effect cannot be due to their well-known intrinsic detergent and hemolytic properties.

Quaternary ammonium compounds are reported to penetrate the erythrocyte membrane with difficulty, first getting into the outer leaflet of the lipid bilayer and then gradually translocating into the inner leaflet [18]. Thus, DM, HC3, and DTMA could act by inserting themselves into the erythrocyte bilayer and modifying the membrane properties of the host cell. To test this, erythrocytes were incubated for 48 hr with 2×10^{-5} M HC3, 5×10^{-5} M DM or 3×10^{-6} M DTMA, concentrations totally inhibiting *P. falciparum* growth (cf. Fig. 1). No modification of erythrocyte capacity for infection was further observed. They supported normal growth of *P. falciparum*, suggesting that the antiplasmodial effect of these compounds is not linked to a mere alteration of host cell membranes.

We were interested in further determining the mechanism of action of these quaternary ammonium containing compounds: does the antiplasmodial effect of these analogs result, as expected, from a specific alteration of PL metabolism, rather than, for instance, from a generalized disturbance of parasite metabolism?

DM, HC3, and DTMA were tested on PL metabolism, via the incorporation of [methyl-^{14}C]choline (Amersham) into PC, as well as on DNA and protein biosynthesis in *P. knowlesi*-infected erythrocytes, which can be obtained in large amounts and with high parasitemia from infected monkeys. Figure 2 clearly shows that DM and HC3 specifically curtail PC biosynthesis starting at 6×10^{-6} and 2×10^{-5} M, respectively, without any modification of protein or DNA biosynthesis. At 2×10^{-5} M, DTMA specifically altered the biosynthesis of PC whereas at high concentrations and with long preincubation times, the viability of the parasite was rapidly affected, since macromolecule biosynthesis decreased in parallel.

Further investigations were carried out to determine whether any biosynthetic pathway of PL other than PC was also affected by the quaternary ammonium compounds. Infected cells, preincubated for 60 min in the presence of the drug, were incubated for 90 min with various radioactive PL precursors: glycerol, oleic acid, serine or choline (results not shown). In the presence of DM or HC3 up to 2×10^{-4} M, none of the precursors, except choline, showed any significant differences in incorporation into PL. At 10^{-5} M, DTMA also selectively altered the PC Kennedy pathway. At 4×10^{-4} M, however, a severe decrease in all PL biosynthesis corresponded to the death of the parasite, as previously seen in protein or nucleic acid incorporation in Fig. 2.

To determine the way in which these compounds selectively alter PC biosynthesis, we studied the incorporation of [^{14}C]choline into the various metabolites leading to PC inside infected erythrocytes (intracellular choline, phosphorylcholine, CDP-choline, PC). Starting at 6×10^{-6} M, DM decreased intracellular radioactive choline even after a short preincubation of 30 min. Radioactive phosphorylcholine, as well as PC, was concomitantly decreased, with 70% inhibition at 4×10^{-4} M. The same profile of inhibition was observed with HC3 and DTMA at 2×10^{-5} M (results not shown). On the basis of these studies, we have tentatively concluded that only an action of the drugs on choline transport into erythrocytes and/or its phosphorylation by choline kinase can be involved in the decreased PC biosynthesis.

We have shown that choline kinase is a soluble parasite specific enzyme whose K_m for choline is 79 ± 20 μM (submitted for publication). DM, HC3 and DTMA are quite weak competitive inhibitors of the enzyme since their K_I are 7–20 times higher than the K_m for choline. Furthermore, quaternary ammonium compounds would have to enter the erythrocytes to have access to parasite choline kinase. This hypothesis is unlikely if we refer to normal uninfected erythrocytes [8] since these amphiphile cationic compounds are reported to have difficulty crossing the membrane. On the other hand, these quaternary ammonium compounds,

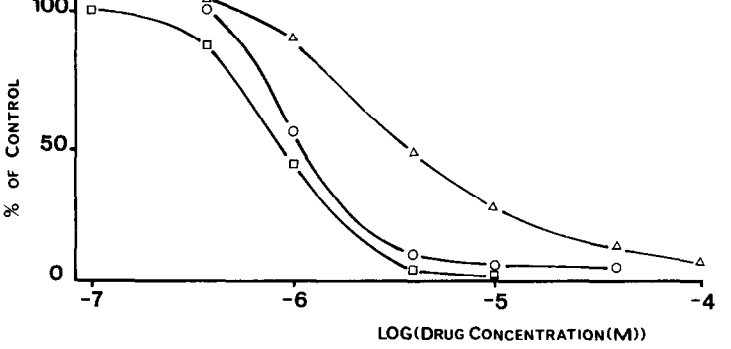

Fig. 1. Effects of quaternary ammonium compounds on *P. falciparum* growth. Drugs were applied for 48 hr to *P. falciparum*-infected cells (0.2–0.3% initial parasitemia, 7% hematocrit), and then media were replaced by fresh medium. On day 4, parasitemias of control and treated samples were monitored on blood smears using a Giemsa stain [7]. Each experiment with various concentrations of drugs was performed in triplicate at least twice. Parasitemias on day 4 are expressed as percentages of control (6–13% depending on the experiment). Hemicholinium 3 (△), decamethonium (○), decyltrimethylammonium (□).

which are well known inhibitors of the choline transport system in erythrocytes [9, 19, 20] without crossing the erythrocyte membrane [8], effectively decrease the intracellular radioactive choline.

Thus, these choline quaternary ammonium compounds specifically interfere with PC metabolism by blocking choline transport into infected erythrocytes and inhibit *P. falciparum* growth *in vivo*. Hence, choline transport is a crucial process for parasite growth and could be an ideal target for a new chemotherapeutic approach to malaria. The supply of this precursor by the lipid component of plasma lipoproteins, which are modified in malaria-infected Mammalians [21], seems unlikely [6] and remains to be demonstrated. We are, however, fully aware that these kinds of molecules would also interfere with the central and peripheral nervous systems. Further investigation is needed to determine to what extent the choline transport system into erythrocytes is modified after infection by *Plasmodium*. Since a significant increase in PL content occurs after infection, it can be expected that an increase in choline

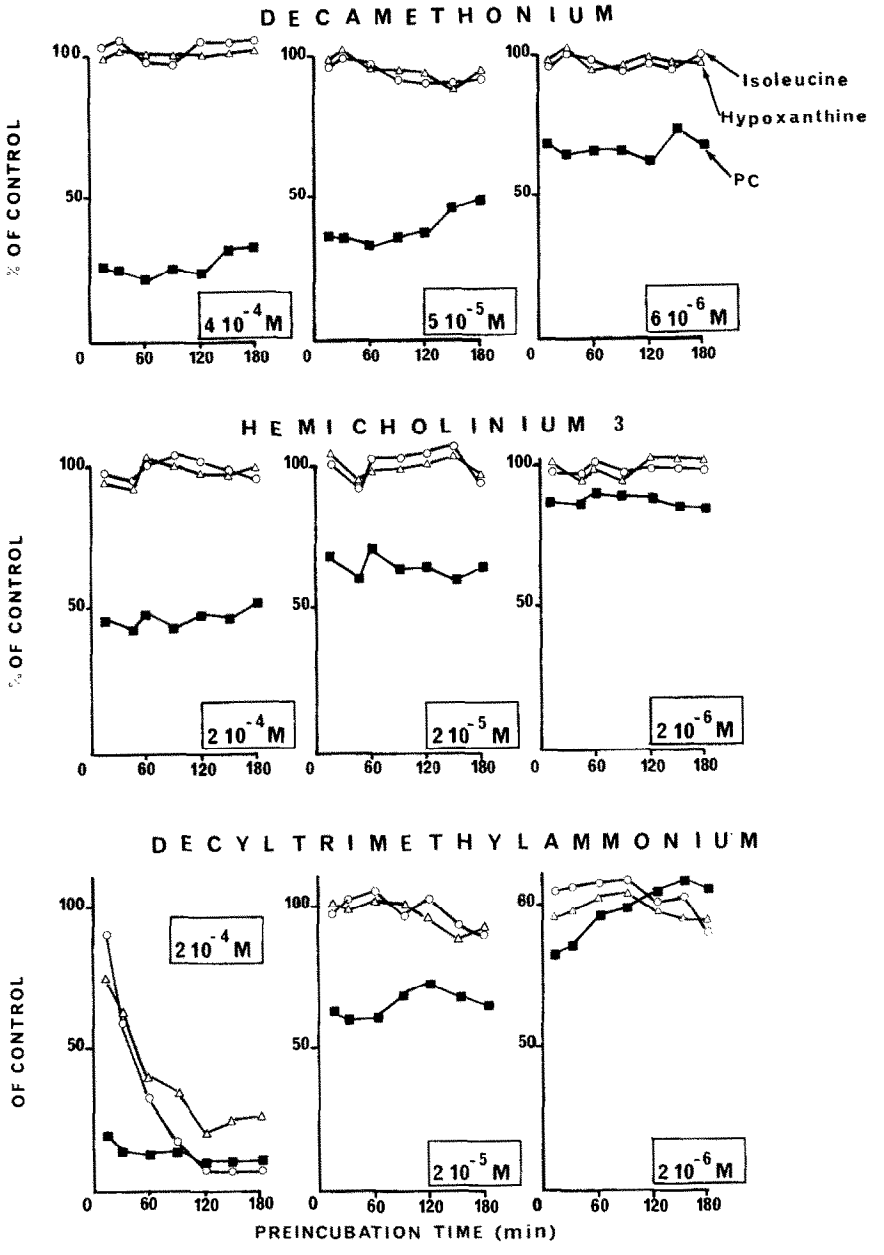

Fig. 2. Effects of quaternary ammonium compounds on the incorporation of labeled hypoxanthine and isoleucine, into TCA-insoluble material and of labeled choline into PC of *P. knowlesi*-infected erythrocytes. Preincubations were carried out at 15% hematocrit in modified RPMI containing the indicated concentrations of drug. Enriched medium with 17.0 Ci/ml [^{3}H]hypoxanthine (△), 4.2 Ci/ml [^{14}C]isoleucine (○), or 0.7 Ci/ml [^{14}C]choline (■) was then added and cells were further incubated for 30 min. The control corresponds to cells preincubated without drug. Each point represents the mean of triplicate samples. Decamethonium (DM) was in contact with 2.5×10^8 infected cells at 24.5% parasitemia, hemicholinium 3 (HC3) with 1.4×10^8 infected cells at 19.7% parasitemia, decyltrimethylammonium (DTMA) with 5.9×10^8 infected cells at 69% parasitemia.

incorporation stems from a transport system activation or even from a modification of this carrier.

In summary, quaternary ammonium-containing compounds were assayed for their inhibiting capacities on *P. falciparum* growth *in vitro*. Hemicholinium 3, decamethonium and decyltrimethylammonium were efficient inhibitors, with IC_{50} of 4×10^{-6}, 10^{-6} and 7×10^{-7} M, respectively. They specifically inhibited phosphatidylcholine biosynthesis without altering the biosynthesis of other phospholipids, proteins, or nucleic acids. This effect was clearly related to an impairment of choline entry into erythrocytes.

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In vitro DNA methylation by methylnitrosourea in isolated copper- or silver-preloaded rat liver nuclei

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The administration to rats of copper in the diet has been reported to suppress tumorigenesis in the liver [1] and kidney [2] by dimethylnitrosamine (DMN). However, the mechanism of action of copper against tumorigenesis in DMN-treated rats has not been studied adequately. We recently reported [3] that the subcutaneous injection of cupric acetate has a suppressive effect on tumorigenesis in the liver of DMN-treated rats. Under our experimental conditions, no tumor was observed in the kidney. We also showed that the methylation of liver DNA in rats given a single dose of DMN is suppressed significantly by copper administration. It was reported that the cytochrome P-450 content of liver microsomal fractions is decreased by the administration of cupric ions [4]. Therefore, the possibility exists that the administration of copper decreases DMN *N*-demethylase activity, leading to a decrease of DNA methylation and tumorigenicity in the liver of rats given DMN. We are studying whether the administration of copper alters DMN *N*-demethylase activity in the liver. Furthermore, we presume that the *in vivo* binding of copper to DNA protects the modification of DNA by DMN as an initiation step of carcinogenesis, because cupric ions and DMN as the methylating compound, which are recognized as electrophilic reactants, are known to react with nucleophilic sites in DNA *in vitro* and *in vivo*. Cupric ions are also known to prefer the GC regions of DNA [5]. There is the possibility, therefore, that the binding of cupric ions to guanine sites in DNA interacts with the modification of guanine by DMN. A selective reaction of silver ions with the GC regions of DNA has also been described [6]. By the administration of silver to rats, the binding of silver ions to the guanine sites in DNA may interact with the modification of guanine by DMN, although there is no convincing evidence that administration of silver to rats suppresses liver tumorigenesis by DMN. The methylation by DMN or methylnitrosourea (MNU) of guanine in DNA in copper- or silver-preloaded rat liver nuclei has not been clarified.

Jensen [7] has reported that the rate of DNA methylation *in vitro* with DMN activated by microsomal fractions correlates well with the rate of formaldehyde formation, indicating DMN *N*-demethylase activity. He also found 7-methylguanine (7MeG), 3-methyladenine and O^6-methylguanine (O^6MeG) in the DNA hydrolysates in the same relative proportions as observed in DNA isolated from similar incubation mixtures containing MNU as the methylating compound.

In this paper, we examine the *in vitro* methylation of guanine in DNA in isolated copper-preloaded or silver-preloaded rat liver nuclei by MNU, instead of DMN which requires activation by microsomes.

Materials and methods

Chemicals. MNU containing ^3H in the methyl group was purchased from the New England Nuclear Corp., Boston, MA (1.0 Ci/mmole). 7MeG was from the Sigma Chemical Co., St. Louis, MO. O^6MeG was synthesized in our laboratory according to the method of Balsiger and Montgomery [8]. All other reagents used in this study were of special grade.