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RAPID ACTION OF QINGHAOSU AND RELATED DRUGS ON INCORPORATION OF [^{3}H]ISOLEUCINE BY *PLASMODIUM FALCIPARUM IN VITRO*

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Abstract—Using the incorporation of [^{3}H]isoleucine into acid-insoluble products as an index of protein-synthetic activity, it was shown that Qinghaosu and two related drugs had a rapid effect on this process in human erythrocytes infected with *Plasmodium falciparum in vitro*. Inhibition could be seen 1 hr or less after addition of the drugs at concentrations from 5 μmole/l. to 50 nmole/l. It is recommended that the effects of these drugs be studied in cell-free protein-synthetic systems.

Qinghaosu (artemisinine, QHS) is a novel antimalarial drug (**I**) developed from the traditional Chinese herbal remedy Qinghao (*Artemisia annua* L.) [1]. The plasmodicidal effects have been confirmed in experimental studies in animals, and in clinical trials against both chloroquine-sensitive and -resistant strains of *Plasmodium falciparum*. In order to enhance antimalarial activity and solubility, a series of derivatives have been synthesized. Although much work has been carried out on chemical properties, and some preclinical and clinical studies have been done, little data are available on the mode of action [1–3]. It is known that the endoperoxide linkage is essential for antimalarial activity. Para-aminobenzoic acid does not antagonise the antimalarial action. Morphological studies indicate that vacuolation, atrophy and disappearance of the cytoplasm follow exposure of the intraerythrocytic malaria parasite to the drug.

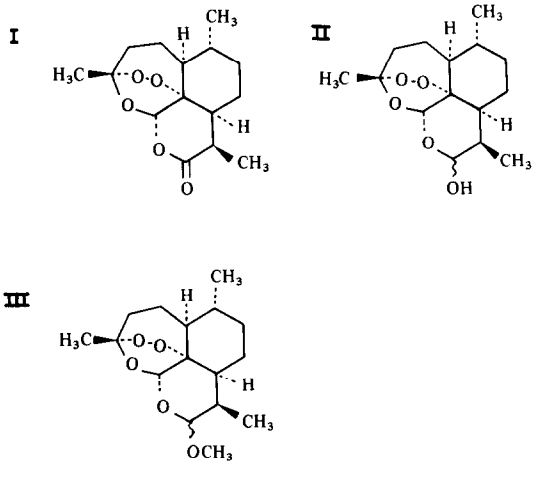
We have earlier investigated the incorporation of [^{3}H]hypoxanthine into nucleic acids by erythrocytes infected with *P. falciparum* (in preparation) and were able to show a delayed inhibition by QHS and related compounds. This observation suggested that nucleic acid synthesis was not the primary target for drug action, and we have therefore studied [^{3}H]isoleucine incorporation into parasitised cells as an index of protein synthesis.

MATERIALS AND METHODS

Drugs. QHS, dihydroqinghaosu (DHQ) (**II**) and artemether (**III**) [4] were dissolved in *N*,*N*-dimethyl formamide (Koch-Light, D.M.F.) and then diluted 4-fold with glucose-enriched culture medium (CM) [5] to the following concentrations: QHS, 5×10^{-4} mole/l.; DHQ, 5×10^{-5} mole/l.; and artemether, 5×10^{-6} mole/l. Cycloheximide (from Sigma) was directly dissolved in CM to give a concentration of 1×10^{-3} mole/l. A control solution consisting of the same amount of DMF in CM as in the diluted drug solutions was made. All the above stock solutions were stored at −20°.

Labelled precursor. L-[4, 5^{3}H]Isoleucine ([^{3}H]Ile) (specific activity 596 mCi/mg) was purchased from Amersham International Ltd. (U.K.) and was supplied as an aqueous solution containing 2% ethanol. The radioactive concentration of the solution was 1.0 mCi/ml. This solution was diluted in CM to give 40 μCi/ml, then filtered through a 0.22 μm membrane for sterilization and stored at −20°.

Parasite cultivation and sampling. The Wellcome/Liverpool/West African strain of *P. falciparum* [6] was used, employing the simplified procedure of Osisanya *et al.* [5]. The haematocrit was 5% and the range of starting parasitaemias was 3–13%. After the addition of [^{3}H]Ile giving a final radioactive concentration of 2 μCi/ml, the cell suspension was divided into aliquots in 50 ml plastic screw-capped cultivation flasks (NUNC, Denmark). The drug solutions under study and the control solution were added as 10 μl/ml culture suspension to



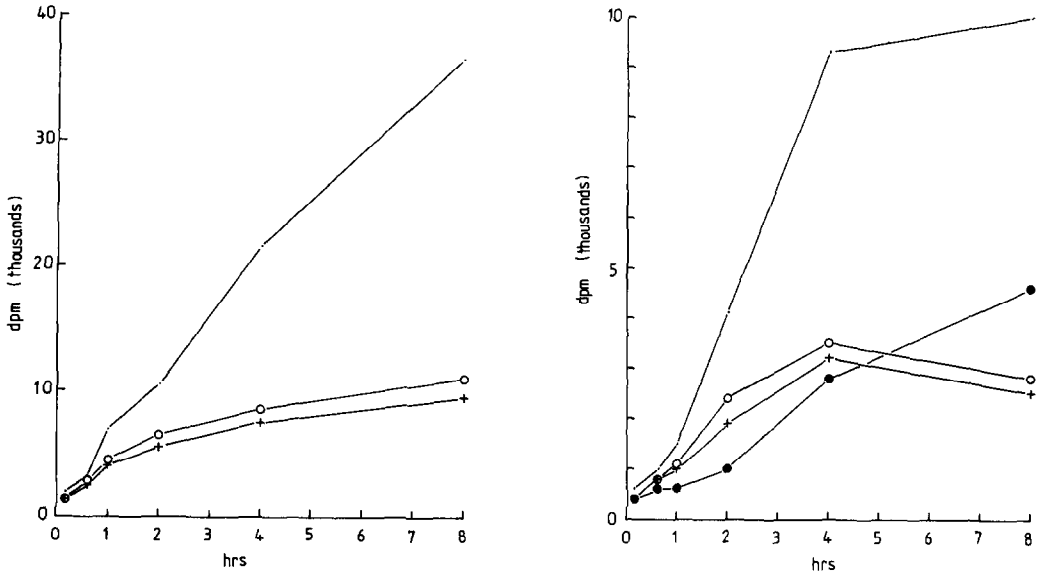


Fig. 1. Parasitaemia 11%. QHS at 5×10^{-6} mole/l. (○). DHQ at 5×10^{-7} mole/l. (+).

Fig. 2. Parasitaemia 5%. QHS at 5×10^{-6} mole/l. (○). DHQ at 5×10^{-7} mole/l. (+). Cycloheximide at 3×10^{-5} mole/l. (●).

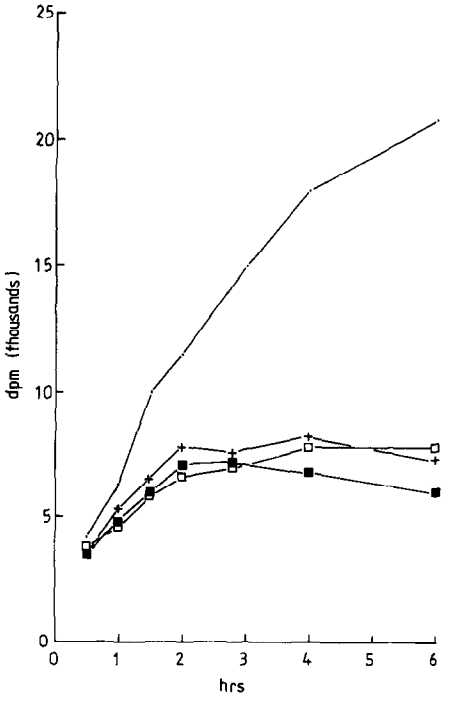


Fig. 3. Parasitaemia 8%. Artemether at concentrations of 5×10^{-6} mole/l. (■), 5×10^{-7} mole/l. (□) and 5×10^{-8} mole/l. (+).

Figs. 1–3. Incorporation of [^{3}H]Ile into protein by *P. falciparum* infected human erythrocytes. Drugs were added to the culture at 0 hr. Erythrocyte concentration was 5%. The control group is denoted by –·– throughout. The dpm illustrated are from cells harvested from 250 μl aliquots of culture suspension.

each flask, and they were incubated at 37° after gassing with 5% O_2, 5% CO_2, 90% N_2. Aliquots (250 μl) of suspension were pipetted into microcentrifuge tubes at the desired time intervals, and stored on melting ice until used. Duplicate samples were employed.

Sample preparation and determination of radioactivity. This was modified from Neame's technique [7]. Samples were removed from the ice and spun in a microcentrifuge at 2000 *g* for 10 sec. The supernatant was discarded and the pellet washed twice by centrifugation in 400 μl of 0.9% NaCl. The pellet was lysed in 200 μl distilled water and the extract was treated with an equal volume of 10% trichloracetic acid. Precipitates were filtered on Whatman 3MM filter paper discs (2.3 cm diameter) on a sintered filter connected to a suction pump, and washed with 15 ml distilled water. The filter papers were put into standard plastic counting vials (Packard) and 150 μl of freshly prepared bleaching solution consisting of equal volumes of glacial acetic acid, ethyl acetate and hydrogen peroxide (all from BDH) was added. The vials were tightly closed and heated in a water bath at 100° for 1 hr. Immediately after heating, 50 μl of concentrated HCl was added to each hot vial, which was then allowed to cool to room temperature. Finally 10 ml of emulsifier scintillator (Packard) was added. The vials were shaken and counting for tritium activity was carried out in a Packard Tri-Carb Scintillation Spectrometer (Model 574). Efficiency was monitored by the external standard, which had itself been calibrated using a tritiated water standard (Amersham). Cpm were corrected after subtraction of background to dpm.

RESULTS

[^{3}H]Ile incorporation by parasitised erythrocytes in controls without drugs was proportional to the parasitaemia (Table 1) and incubation time (Figs. 1–3). Better incorporation was observed in the first 4 hr, after which there was some falling off.

QHS at 5×10^{-6} mole/l. and DHQ at 5×10^{-7} mole/l. caused a marked inhibition of incorporation, beginning less than 1 hr after addition of the drug. The curves of inhibition with time for these two drugs were similar (Figs. 1 and 2). These experiments were repeated with similar results. The results presented in Fig. 2 show that cycloheximide at 3×10^{-5} mole/l. also had inhibitory effects on [^{3}H]Ile incorporation. Although cycloheximide had a marked effect in the first 2 hr, this inhibition was reduced with continuing incubation. No such falling off in effect was found with QHS or DHQ. None of the drugs had a detectable effect on parasitaemia although after 8 hr with QHS and DHQ partial disappearance of parasite cytoplasm, with only residual dots remaining, had taken place (Table 2).

Table 1. Relationship between parasitaemia and amount of [^{3}H]Ile incorporation after incubation for 4 hr

Parasitaemia (%)	Developing stage of parasites	Incorporation in cells from 250 μl sample (dpm)
3	Later ring forms	2653
5	Developing schizonts	9386
8	Developing schizonts	17,853
11	Developing schizonts	21,719
13	Developing schizonts	24,308

Table 2. Parasitaemia before and after incubation*

Group	Parasitaemia (%)	
	Before incubation	After incubation for 8 hr
Control	13.0	15.1
QHS	13.0	14.4
DHQ	13.0	15.2
Cycloheximide	13.0	13.0

* The division cycle of *P. falciparum* takes 48 hr. Antimalarials in general have little effect on parasitaemia *in vitro* over 8 hr.

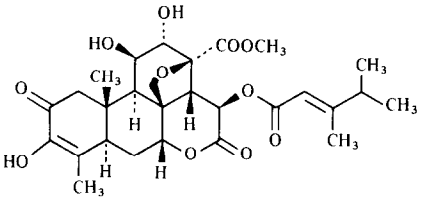
A further experiment was carried out using another QHS derivative, artemether [4] and at the concentrations tested it had a similar effect and time course of inhibition to that shown by QHS. Even at 5×10^{-8} mole/l. this drug showed a maximal effect (Fig. 3).

DISCUSSION

Isoleucine is an essential exogenous amino acid for protein synthesis by malaria-infected erythrocytes [8]. The amount of [^{3}H]Ile incorporation is therefore an index of protein-synthetic activity. The effects of the eukaryotic protein synthesis inhibitor cycloheximide on labelled Ile incorporation into protein have been reported before in *P. falciparum* [9] and *P. lophurae* [10]. Cycloheximide was found to inhibit chloroquine-induced pigment clumping in *P. berghei in vitro* at concentrations similar to those used here on *P. falciparum* [11], and this drug was found to inhibit the growth of *P. knowlesi* ring forms to schizonts *in vitro* [12].

A possible explanation for the anomalous time-course curve for cycloheximide is that since this drug inhibits eukaryotic ribosomes specifically [13], some of the protein synthesis occurring after 4 hr may be on the prokaryotic ribosomes of the plasmodial mitochondria. It is also possible that this inhibitor was being slowly inactivated during incubation.

We have shown that even low concentrations of QHS-like compounds inhibit protein synthesis after only 1 hr incubation. In other experiments, to be described elsewhere, 2 hr exposure was needed to demonstrate inhibition of [^{3}H]hypoxanthine incorporation into nucleic acids with the same strain of parasite and the same drug concentrations. Moreover, the extent of inhibition of protein synthesis by QHS-like compounds was greather than the inhibition of nucleic acid synthesis. This suggests that the inhibitory action exerted by QHS-like compounds may be direct, and not simply secondary protein synthesis inhibition following on effects on other systems. It is now important to study the effects of QHS and derivatives on cell-free protein synthesis.



IV

QHS has structural similarities to the quassinoids, simalikilactone, glaucarubinone, soularubinone and bruceantin (**IV**), which are potent antimalarials *in vitro* [14, 15]. Bruceantin is known to inhibit protein synthesis on mammalian ribosomes at micromolar concentrations [16].

If QHS and related drugs prove to be specific inhibitors of protein synthesis in malaria parasites, they may be of importance in the development of drug combinations to combat emerging resistance to current antimalarial drugs. This is of especial interest in view of the potentiation of chloroquine's activity on chloroquine-resistant strains of *P. berghei* by erythromycin, another inhibitor of protein synthesis [17].

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