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Plasmodium falciparum: effects of amantadine, an antiviral, on chloroquine-resistant and -sensitive parasites *in vitro* and its influence on chloroquine activity

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Abstract—The lysosomotropic nature of amantadine suggested potential as an antimalarial. Sensitivity tests to amantadine hydrochloride alone and in combination with chloroquine were carried out in 96-well microtitre plates using the tritiated hypoxanthine uptake method to measure parasite growth. Amantadine alone has antimalarial activity. Amantadine is more potent against chloroquine-resistant strains. Combinations of amantadine and chloroquine result in slight synergy in both resistant and sensitive strains.

Amantadine (1-aminoadamantane) is a primary amine that has both prophylactic and therapeutic efficacy against type A influenza virus infections and is a treatment for Parkinson's disease [1]. Amantadine is a amphiphilic weak base ($pK_a = 9$), structurally unrelated to the quinolines. Based on its amphiphilic nature amantadine is expected to penetrate in its uncharged form and rapidly load the cytosol to a concentration of the order of the extracellular concentration, then accumulate within the acidic organelle, the food vacuole, to much higher concentrations trapped in the hardly permeable protonated form [2].

The aim of this study was to examine the effect of amantadine alone and in combination with chloroquine on the intraerythrocytic growth of *Plasmodium falciparum*.

Materials and Methods

Chemicals. Amantadine chloride salt and chloroquine diphosphate salt were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). [^{3}H]Hypoxanthine was obtained from Amersham (U.K.). All other chemicals were of the best available grade.

Parasite. Strains of *P. falciparum* used included the chloroquine-sensitive isolate RSA-2 obtained from J. Freese (RIDTE, R.S.A.) and the chloroquine-resistant strains FCR-3 obtained from J. Freese and 7G8 obtained from D. Walliker (Edinburgh, U.K.). The strains were maintained in O$^+$ blood in RPMI-1640 medium (Highveld Biological, R.S.A.) supplemented with 10% human serum, 25 mM HEPES, 25 mM $NaHCO_3$ in accordance with the method of Freese *et al.* [3].

Determination of inhibitory concentrations of each drug alone. Drug sensitivity tests were carried out according to the method of Desjardins *et al.* [4]. Briefly, tests were carried out in 96-well microtitre plates; 25 μL of drug appropriately diluted were added to each well. To this were added 200 μL of a synchronous (in the early ring stage) parasitized erythrocyte suspension [5] to give a 0.5% parasitemia and 1% haematocrit in each well. Tritiated hypoxanthine (0.2 μCi) was added to each well after 24 hr incubation, then cells were harvested after a further incubation period of 18 hr using a semi-automatic cell harvester. The dried discs were processed for scintillation counting and counted in a scintillation spectrometer. Counts were corrected for incorporation of [^{3}H]hypoxanthine into uninfected and non-drug-treated cells. IC_{50} values (representing the molar concentration resulting in 50% decrease in [^{3}H]hypoxanthine incorporation compared to drug-free controls) were obtained for each drug using a computer programme (Enzfitter®).

Determination of the effect of amantadine on the IC_{50} of chloroquine. The effect of amantadine on the IC_{50} values of chloroquine in the different lines of *P. falciparum* was determined using the method of fixed ratios as described by Martin *et al.* [6]. The results were plotted as isobolograms.

Results and Discussion

The values given in Table 1 verify that the FCR-3 and 7G8 strains are chloroquine resistant and the RSA-2 strain is chloroquine sensitive. Amantadine was found to have antimalarial activity *in vitro*. As seen in Table 1, amantadine is more potent against the resistant strains than the sensitive strains, suggesting it may somehow react with the mechanism involved in conferring chloroquine resistance, which is widely believed to be related to changes in drug transport rather than the mechanism of action [7].

The lysosomotropic hypothesis suggests that the selective accumulation of amantadine in the acidic organelle results in alkalinization [8]. The complete degradation of macromolecules within the food vacuole depends on the maintenance within the organelle of an acidic pH, since it is performed by enzymes with a low pH optima. The slight elevation of the pH could block vacuolar digestion of host cell haemoglobin with consequent parasite starvation [9].

Amantadine, in addition to its lysosomotropic nature, is a membrane-active drug. Amantadine is believed to be incorporated into membranes in the head group region. At neutral and acidic pH values, amantadine molecules have a charge of +1; therefore, on adsorption to the negatively charged membrane surface the drugs induce a negative electrical potential difference on the membrane. The change in membrane surface charge density causes subsequent changes in the electrostatic free energy of the bilayer and influences the phase behaviour of the lipid molecules and lipid–protein interactions, and consequently the fluidity and mechanical bending properties of the membrane, and thereby membrane–membrane interactions such as adhesion, fusion and exo- and endocytosis [10].

Table 1. The IC_{50} values for chloroquine and amantadine for different strains of *P. falciparum* as determined by inhibition of radiolabelled hypoxanthine incorporation using synchronous cultures (ring stage), starting with 0.5% parasitemia and 1% haematocrit

Strain	IC_{50} chloroquine (CQ) (nM)	IC_{50} amantadine (AMA) (μM)	Ratio AMA/CQ
FCR-3	151.49 ± 2.59	5.35 ± 1.15	35.32
7G8	65.54 ± 1.7	33.72 ± 6.2	514.49
RSA-2	19.82 ± 0.28	188.44 ± 11.3	9507.57

Figures represent mean estimates of IC_{50} derived from dose–response curves performed in triplicate ± standard error.

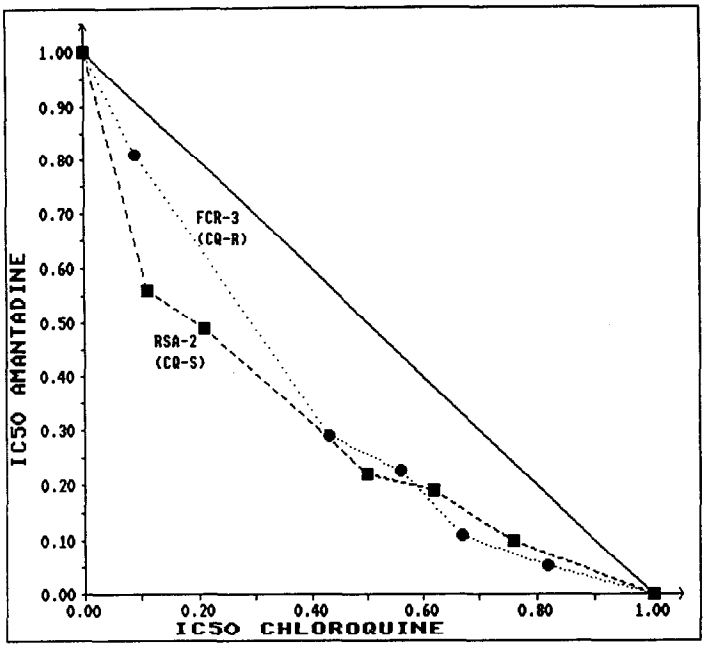

Fig. 1. Isobologram showing the effect of using combinations of chloroquine and amantadine in FCR-3 (CQ-resistant CQ-R) and RSA-2 (CQ-sensitive CQ-S) strains. Control IC_{50} for amantadine and chloroquine was normalized to one unit. Each drug combination IC_{50} was plotted as a fraction of the control.

Membrane-bound receptors are sensitive to the structural and physical properties of the lipid bilayer; changes may lead to functional changes in receptor-mediated responses or receptor availability [11, 12] within the parasite. The high concentration of amantadine in the lysosome coupled with its liphophilicity could cause disruption of the lysosomal membrane and a release of hydrolases into the cytoplasm, causing cell death or leakage of ions through the lysosomal membrane and resulting in alkalinization of the food vacuole [13].

Amantadine in combination with chloroquine was found to be slightly synergistic in both chloroquine-resistant and -sensitive strains (Fig. 1). Amantadine does not reverse chloroquine resistance of *P. falciparum* since it increases the effect of chloroquine on both resistant and sensitive strains, while drugs with reversal activity such as verapamil enhance the schizontocidal effect in chloroquine-resistant strains only [6, 14]. This result is surprising since amantadine and chloroquine are expected to compete for H^+ ions [15]. Recent evidence [16] suggests that interactions with hydrogen bonding groups within the polar regions of the membrane are important in the membrane transport of chloroquine. Changes in the fluidity could change the permeability coefficient of chloroquine resulting in increased uptake [17], accounting for the observed synergism.

In addition, amantadine depresses hepatic microsomal metabolism of aminopyrine (N-demethylation) in rats [18]. These enzymes are believed to be responsible for chloroquine detoxification. *In vitro* studies [19] have demonstrated N-demethylation of aminopyrine activity in *P. falciparum* and demonstrated a relationship between enzyme activity and resistance. Amantadine–chloroquine synergism may be a result of inhibition of chloroquine breakdown.

Amantadine's action appears to be more selective toward the chloroquine-resistant strains of *P. falciparum* and when combined with chloroquine it is slightly synergistic in both sensitive and resistant strains. These antimalarial properties are exhibited at levels that are attainable *in vivo* in man: oral administration of 100 mg twice daily results in a steady-state concentration of 0.1–1.1 μg/mL (0.6–7.3 μM) [20]. We believe, due to its distinctive relationship with chloroquine resistance, amantadine shows promise as a tool to shed light on the mechanism(s) of chloroquine resistance.

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