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The toxicity of amodiaquine and its principal metabolites towards mononuclear leucocytes and granulocyte/monocyte colony forming units

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The cytotoxicity of amodiaquine (AQ), amodiaquine quinoneimine (AQQI) and desethyl-amodiaquine (AQm) has been assessed in comparison with that of chloroquine (CQ) using mononuclear leucocytes (MNL) and granulocyte/monocyte colony forming units (GM-CFU) from haematologically normal subjects. Toxicity toward MNL was assessed after 2 h and 16 h incubations with each compound. After 2 h, AQ, AQm and AQQI but not CQ (within the concentration range 1–100 μmol l^{-1}) produced a significant decrease in cell viability. After 16 h, all four compounds significantly increased cell death. After both 2 h and 16 h incubations CQ was the least toxic and AQQI the most toxic of the four compounds towards MNL. Toxicity to GM-CFU was assessed by the inhibition of colony formation *in vitro*. After 10–14 days incubation, there was significant concentration-dependent inhibition of colony formation by AQ, AQm, AQQI and CQ (within the range 0.1–10.0 μmol l^{-1}). There were no significant differences between the ability of the four compounds to inhibit colony formation but toxicity towards GM-CFU was observed at drug concentrations at least 10-fold lower than those that were toxic to MNL. These data show that the four compounds are equally toxic *in vitro* toward GM-CFU, although some differences in their toxicity toward MNL were seen. The possible mechanisms of AQ's toxicity are discussed.

Keywords amodiaquine chloroquine cytotoxicity

Introduction

In the early 1980s amodiaquine (AQ), a 4-amino-quinoline antimalarial drug, was recommended as a chemoprophylaxis for travellers to areas of chloroquine (CQ)-resistant malaria. This followed the demonstration that AQ is superior to CQ against some strains of CQ-resistant *Plasmodium falciparum* (Watkins *et al.*, 1984). Unfortunately, the prophylactic use of AQ carries a high risk of agranulocytosis and hepatitis, and several deaths resulted (Hatton *et al.*, 1986; Neftel *et al.*, 1986); consequently AQ is no longer used prophylactically. However, since AQ is effective and cheap and because no cases of serious toxicity have been reported during its use in acute malaria, AQ is still frequently used in some countries. Hence its mechanism of toxicity is still of clinical importance.

The effects of AQ on the proliferation of granulocyte-monocyte colony forming units (GM-CFU) obtained from patients with AQ-induced agranulocytosis have been reported (Lind *et al.*, 1973; Rhodes *et al.*, 1986). The maximum concentration of AQ tested in these studies was 0.5 μg ml^{-1} (approximately 1.0 μmol l^{-1}). Cells from patients recovering from AQ-induced agranulocytosis, but not those obtained from healthy control subjects, displayed significant inhibition of colony formation with

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AQ at 0.05–0.5 μg ml^{-1} (approximately 0.1–1.0 μmol l^{-1}). This suggested that patients had developed agranulocytosis as a result of abnormal sensitivity of their myeloid precursors to the drug, and that the myelosuppression was dose-dependent and not idiosyncratic (Lind *et al.*, 1973).

However, at the time of previous investigations of AQ-induced agranulocytosis the clinical pharmacology of the drug was not understood. Following its oral administration, AQ is rapidly and extensively metabolised to a pharmacologically active metabolite, desethylamodiaquine (AQm) (Churchill *et al.*, 1985; Winstanley *et al.*, 1987), and the plasma concentration of the parent drug is low, and indeed may not be detectable in many subjects (Pussard *et al.*, 1987). Other metabolites of AQ are detectable in the plasma after oral administration (Churchill *et al.* 1986; Mount *et al.*, 1987), but none seems to play a major therapeutic role, and none has been implicated as important in the mechanism of toxicity.

In addition to its enzymic biotransformation, AQ undergoes non-enzymic autoxidation in neutral aqueous solutions, yielding a protein-arylating quinoneimine (AQQI) (Maggs *et al.*, 1987, 1988). The toxicity of other drugs, most notably paracetamol, has been attributed to quinoneimine derivatives (Tee *et al.*, 1987), and it seemed that the formation of such a reactive species in marrow cells and hepatocytes could be responsible for the adverse reactions associated with AQ. The concentration of AQ achieved in human bone marrow is not known, but after oral administration of [^{14}C]AQ to rats, radioactivity accumulates in the red marrow (Winstanley *et al.*, 1988).

Consequently, the toxicity of AQ, its principal plasma metabolite AQm and its autoxidation product AQQI have been assessed and compared with that of CQ. Like AQ, CQ is a 4-aminoquinoline but lacks a phenolic moiety and is therefore incapable of autoxidation to a quinoneimine; agranulocytosis is only rarely caused by CQ.

Methods

Materials

Amodiaquine dihydrochloride dihydrate and desethylamodiaquine hydrochloride were supplied by Parke-Davis (Pontypool, UK), and chloroquine sulphate was obtained from Rhone-Poulenc (Dagenham, UK). Amodiaquine quinoneimine was synthesised and characterized as described by Maggs *et al.* (1988). *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES) was obtained from BDH chemicals (Poole, UK). Culture medium (RPMI-1640) was obtained from GIBCO Laboratories (Uxbridge, UK). Batches of foetal calf serum were tested for their ability to support GM-CFU growth; Seralab (Crawley Down, UK) batch number 301121 proved optimal and was used throughout. Bacto agar was supplied by DIFCO Laboratories (Detroit, USA) and human serum albumin (HSA) by Sigma (Poole, UK). The human bladder carcinoma cell line C5637 was a gift from Dr E. Rhodes (Department of Haematology, University of Liverpool). Density gradient medium (Lymphoprep) was supplied by Nycomed (Oslo, Norway).

Stock solutions

For the MNL studies, stock solutions (1.0 mg ml^{-1}) of all four compounds were prepared in absolute ethanol. For the GM-CFU culture studies stock solutions (1.0 mg ml^{-1}) of AQ, AQm and CQ were prepared in sterile distilled water, while the stock solution of synthetic AQQI (2.0 mg ml^{-1}) was prepared in ethanol. All stock solutions were stored in foil-wrapped plastic containers at −20° C, and none was sterilized. The purity of AQ, AQm and CQ was checked by h.p.l.c. (Winstanley *et al.*, 1987), while that of AQQI was checked by t.l.c. and h.p.l.c. The AQQI stock required replacement approximately every 6 weeks. Dilutions were prepared using the culture media, and were made up on the day of each experiment.

Isolation of cells

Peripheral blood MNL were isolated from fresh heparinized venous blood from five healthy subjects as described by Riley *et al.* (1988).

Bone marrow (5 ml) was obtained by percutaneous aspiration from the posterior iliac crests of haematologically normal patients while they were under general anaesthetic prior to surgery. Patients were informed that marrow was to be drawn for research purposes, and gave written informed consent in the presence of an impartial witness. Approval for the study was given by the Ethics Committees of the Royal Liverpool and Broadgreen hospitals. Following aspiration, marrow was transferred to a sterile tube containing heparinized culture medium (2.0 ml). Low-density marrow cells were then isolated as follows: to bone marrow in culture medium (7 ml) was added phosphate buffered saline (PBS; 1:1 v/v) under conditions of laminar-flow sterility. This suspension was carefully layered onto density

gradient medium (1:1 v/v), and was centrifuged (750 *g*; 15 min). Low-density cells in the 'buffy coat' and density gradient medium were isolated, washed twice with PBS, resuspended in culture medium, and counted (50 μl aliquot) by light microscopy.

MNL studies

Peripheral blood MNL ($1–1.5 \times 10^6$) in HEPES buffered medium (1.0 ml; pH 7.4; Spielberg, 1980) were incubated under air with AQ, AQm, AQQI or CQ (1.0, 10.0 and 100 μmol l^{-1}; 3 replicates) in polystyrene tubes at 37° C for 2 h. Controls comprised identical cells incubated in medium containing ethanol (2% v/v). Incubates were then centrifuged (300 *g*; 10 min), the supernatant decanted and the cells resuspended in HEPES-buffered saline containing HSA (5 mg ml^{-1}). Samples were then incubated overnight at 37° C in an incubator.

Cell death was assessed by trypan blue exclusion 2 h and 16 h after the start of incubation. To reduce observer bias, tubes were encoded by another worker, the code being broken upon completion of counting. Trypan blue (0.2 mg ml^{-1}; 25 μl) was added to an aliquot of the cell suspension (100 μl) and approximately 200 cells were examined immediately using a Neubauer counting chamber (Weber Scientific International, Lancing, UK).

GM-CFU culture studies

Bone marrow cell culture was essentially by the method of Myers *et al.* (1984), but using RPMI-1640 and not Alpha medium. Bone marrow (5.0 ml) was obtained from five subjects on separate days, and each of the four compounds was tested on each marrow sample. Low density cells (2×10^5) were suspended in semi-solid medium (RPMI-1640, 1.0 ml) containing foetal calf serum (20% v/v), L-glutamine (2.0 mmol l^{-1}), gentamicin (40 μg ml^{-1}) and agar (0.3% w/v). The cell suspensions were transferred to sterile plastic Petri dishes (35 mm diam.). Conditioned medium from the C5637 cell line (Myers *et al.*, 1984) was used (10% v/v) as the source of granulocyte-monocyte colony stimulating factor (GM-CSF). The effect of the compounds added at the start of cultures on GM-CFU proliferation was tested at 0.1, 1.0 and 10.0 μmol l^{-1}. Control plates for AQ, AQm and CQ comprised drug-free medium, whereas those for AQQI comprised drug-free medium containing ethanol in a proportion identical to that in the 10.0 μmol l^{-1} AQQI plates (0.2% v/v). Four replicates were employed for both control and test plates.

Low density marrow cells from a sixth patient were incubated with the four compounds at the same concentrations as previously, but after 1.0 h the cells were washed with culture medium prior to plating out. Control plates of AQ, AQm and CQ comprised identical cells incubated in drug-free medium prior to washing; control plates for AQQI comprised identical cells incubated in drug-free medium containing ethanol (0.2% v/v) prior to washing; as before, four replicates were employed.

Plates were incubated at 37° C in an atmosphere containing 5% CO_2 for 10–14 days. Plates were encoded by another worker, the code being broken after colony numbers had been counted. All plates from an experiment were inspected, by inverted light microscope, on the same day. The entire surface of each plate was examined, and colony numbers scored manually. Clusters of > 40 cells were counted as colonies (Pike & Robinson, 1970).

Statistical methods

In each experiment the data obtained for each treatment (3 replicates in MNL studies, 4 replicates in GM-CFU studies) were averaged and the average values obtained from each of five separate experiments were analysed by one-way analysis of variance, and subsequently, where appropriate, by the unpaired Student's *t*-test.

Results

MNL studies

At the start of each experiment MNL cell viability was > 99% in control incubates. In the absence of drug, cell viability after 2 h of incubation was 98.9 ± 0.4% and after the 16 h incubation period was 98.9 ± 0.5% (mean ± s.d., $n = 5$ experiments). When MNL were incubated for 2 h with each of the four test compounds AQ, AQm, AQQI but not CQ inhibited cell viability (Figure 1a) to an extent that was statistically significant (P values 0.002, < 0.001, < 0.001 and 0.056 respectively for the four compounds by one-way analysis of variance). When the cells were washed after the 2 h incubation and resuspended in drug-free medium for 16 h, a similar pattern of cell death was seen (Figure 1b): all four compounds produced a significant degree of cell death ($P = 0.001$, < 0.001, < 0.001 and 0.008 for AQ, AQm, AQQI and CQ respectively by one-way analysis of variance). There were no differences in the pattern and extent of cell death between the two time periods (2 h and 16 h) with regard to

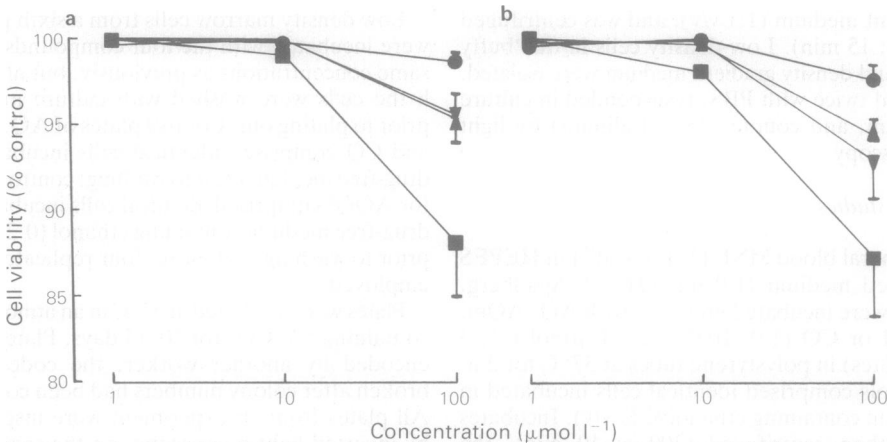

Figure 1 Viability of MNL (% control) after incubation with AQ (▼), AQm (▲), AQQI (■) and CQ (●). Cells were incubated initially for a period of 2 h (a) and were subsequently washed and resuspended in drug-free medium for 16 h (b). Each point is the mean ± s.e. mean of five separate experiments (each performed in triplicate) on cells from different donors.

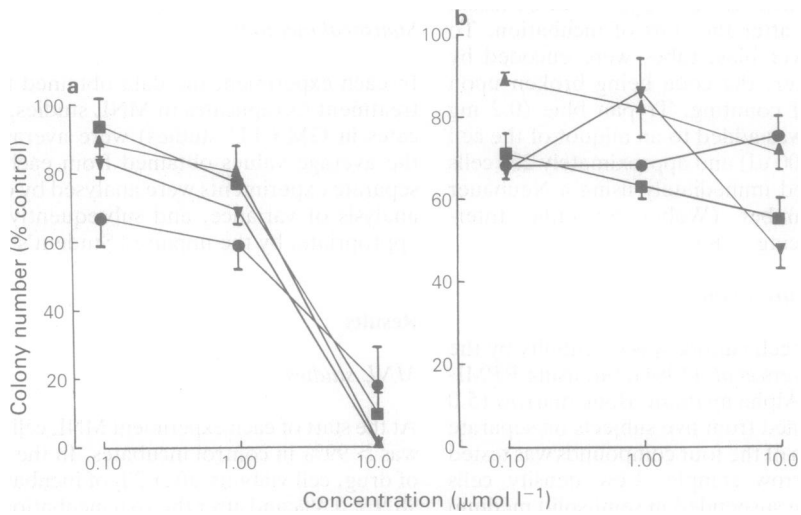

Figure 2 GM-CFU colony numbers (% control) at 10–14 days of culture of bone marrow cells after treatment with AQ (▼), AQm (▲), AQQI (■) and CQ (●). a) The drug was present for the full culture period. Each point is the mean ± s.e. mean of five separate experiments (each performed in quadruplicate) on cells from different donors. b) The cells were treated for 1 h with the compounds, then washed and cultured for 10–14 days. Each point is the mean ± s.e. mean for four replicate cultures of cells from one donor.

cell viability seen with each compound (Figure 1a,b). At both 2 h and 16 h the chemical-induced toxicity was concentration-dependent over the range 1–100 μmol l^{-1}. AQQI produced the maximum observed decrease in cell viability but this effect was subject to some interindividual variation (Figure 1). CQ (100 μmol l^{-1}) was significantly less toxic than AQm after 2 h and AQQI after 2 h and 16 h ($P < 0.05$ for each comparison by the unpaired Student's *t*-test).

GM-CFU culture studies

In control plates granulocyte/monocyte colon numbers ranged from 30–110 (per 2 × 10^5 lo density cells plated), and in the presence c ethanol (0.2% v/v) they ranged from 38–11 Figure 2a shows colony numbers (expressed a percentage of control) resulting from the cultur of GM-CFU from five subjects in the presence c each of the compounds (10–14 days). Each of th

compounds produced a concentration-dependent inhibition of colony formation over the range 0.1–10 μmol l^{-1} (Figure 2a) that was statistically significant ($P < 0.001$ by one-way analysis of variance). No differences were seen between the pattern and extent of inhibition of GM-CFU colony formation by the four compounds (Figure 2a). Figure 2b shows colony numbers resulting from culture of low density marrow cells from a further subject following their incubation for 1 h with the compounds. None of the compounds inhibited colony formation under these conditions.

Discussion

The mechanism of agranulocytosis associated with the prophylactic use of AQ in man is unknown, though earlier investigations (Lind *et al.*, 1973; Rhodes *et al.*, 1986) suggested that an abnormal sensitivity of myeloid precursors to AQ might predispose individuals to this type of reaction. Inter-individual variation in drug disposition and drug-induced responses of the immune system might also play a role (Young & Vincent, 1980). Clearly, the metabolites of the drug could be involved in these mechanisms, and since nothing was known of their toxicity we have studied the general cytotoxicity and myelocytotoxicity of AQ, its principal metabolite AQm, and its quinoneimine (AQQI).

Amodiaquine does not attain high plasma concentrations after oral administration (Winstanley *et al.*, 1987) and may be undetectable in the plasma of some subjects (Pussard *et al.*, 1987). The principal plasma and urinary metabolite of AQ is desethylamodiaquine (AQm), and it seems likely that this compound is responsible for most of the therapeutic effects of the drug (Churchill *et al.*, 1985; Winstanley *et al.*, 1987). AQm is further metabolized by de-ethylation and hydroxylation. In addition to undergoing extensive enzymic transformation, AQ undergoes autoxidation to a chemically reactive quinoneimine (AQQI) in neutral aqueous solutions. This process is not dependent upon cytochrome P450 but is accelerated by the stimulation-dependent oxidative burst of polymorphonuclear leucocytes *in vitro* (Clarke *et al.*, in press). Quinoneimines, such as that of paracetamol (Holme *et al.*, 1984), are thought to be the ultimate toxic metabolites of a number of compounds, and thus it seemed possible that AQQI might contribute to the toxicity of AQ. Chloroquine was included in the study because it is a 4-aminoquinoline which, unlike AQ, is incapable of forming a quinoneimine and does not frequently cause agranulocytosis. Two distinct assessments of toxicity were employed: an estimate of direct, i.e. brief exposure, cytotoxicity was made with MNL, while the clinically relevant inhibition of bone marrow cell growth was measured using GM-CFU cultures.

Peripheral blood MNL are not the targets of AQ toxicity *in vivo* but, amongst human cells, they are easily obtained in large numbers, and have been used by others in the assessment of drug toxicity (Riley *et al.*, 1988; Spielberg, 1980). They allow simple quantitative comparisons of the cytotoxicity of compounds following a brief (2 h) incubation with cells. The short duration of incubation was selected to investigate the direct cytotoxicity of AQQI since quinoneimines rapidly decompose in aqueous media (Novak *et al.*, 1986), hence the direct cytotoxicity of AQQI consequent upon the derivative's extreme chemical reactivity was likely to be partially counterbalanced by rapid decomposition and deactivation.

While AQQI and, to a lesser extent, AQ and AQm reduced MNL cell viability significantly by 2 h, CQ had no significant effect at this time point. All four compounds produced significant cell toxicity after the 16 h incubation period, and again AQQI was the most toxic and CQ the least toxic compound.

Although there were no significant differences between the toxicity of the four compounds towards the growth of bone marrow cells *in vitro*, the data should be interpreted with caution because of the chemical instability of AQQI and the ready autoxidation of AQ and AQm. Part of the AQQI added to the GM-CFU cultures is likely to have been deactivated via arylation of the protein (foetal calf serum) in the medium; 56% of [^{14}C]AQQI (9.4 μM, final) added to a solution of human serum albumin (4.7 mg ml^{-1}) in 0.1M phosphate buffer (pH 7.4) became irreversibly bound to the protein within 30 min (unpublished data). Inhibition of colony formation was observed at lower concentrations than cell death in the direct cytotoxicity assay—suggesting an intrinsically greater sensitivity of GM-CFU than MNL. In man the maximum plasma concentrations of AQ and AQm following oral AQ 10 mg kg^{-1} are approximately 0.1 μmol l^{-1} and 0.3 μmol l^{-1} respectively (Winstanley *et al.*, 1987). After administration of a similar dose of radiolabelled AQ to rats the drug accumulated in red bone marrow to reach a concentration of 3 μmol l^{-1} (Winstanley *et al.*, 1988). This concentration is within the range that we found to be toxic to GM-CFU but not to MNL in the present study. No inhibition was obtained when the GM-CFU were briefly (1 h) exposed to the compounds and then washed prior to their incubation in drug-free medium for 10–14 days. The lack of a direct

effect of AQQI on GM-CFU is surprising, though it may be supposed that the mechanisms of MNL death and inhibition of colony formation are both qualitatively and quantitatively different; cell killing might be expected to require higher drug concentrations. Prolonged exposure of GM-CFU may have been required for significant inhibition because of a slow uptake of the compounds. The greater effect of CQ upon GM-CFU colony formation than on MNL viability is presently unexplained, although this toxicity cannot involve quinoneimine formation. The observation of CQ's myelocytotoxicity *in vitro* did at least indicate that the GM-CFU method is not predictive of the likelihood of bone marrow suppression *in vivo*.

In summary, the present work has shown that both the principal metabolite of AQ in man and a chemically reactive derivative possess clinically relevant cytotoxicity *in vitro*. Nevertheless, their role in the pathogenesis of agranulocytosis remains to be established. Further studies should consider the possible relationships between cellular susceptibility and immune responsiveness to AQ and its metabolites since AQ has been shown to be immunogenic in man and experimental animals (Christie *et al.*, 1989; Clarke *et al.*, 1990).

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