Manipulation of the N-alkyl substituent in amodiaquine to overcome the verapamil-sensitive chloroquine resistance component.

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The successful chemotherapy of human malaria infection is becoming increasingly difficult because of the widespread problem of parasite resistance to currently available antimalarial drugs (40). Parasite resistance to quinoline-containing antimalarial drugs such as chloroquine (CQ) is characterized by an inability of resistant parasites to accumulate as much drug, at steady state, as their susceptible counterparts (3, 13, 22, 38). This resistance phenotype shares similarities with mammalian multidrug resistance, the most striking of which is mammalian multidrug resistance, the most striking of which is resistance with CQ, namely, AQ, to a compound with a cross-resistance pattern which might limit efficacy in vivo, namely, DesAQ. On the basis of the considerations outlined above, we have selected amopyroquine (AMOPYRO) and N-tertbutylamodiaquine (NTBAQ) (Fig. 1) compounds which should demonstrate pharmacological advantages over both AQ and CQ in vivo, for biological evaluation. Both drugs are predicted to show lipophilicity similar to that of AQ at pH 7.2 and therefore should be poor substrates for the VP-sensitive component of aminoquinoline resistance. Furthermore, the chemical alterations introduced into these molecules, i.e., an N-tertbutyl or pyrrolidine group, are metabolically less labile than the N-ethyl group of AQ (29, 31, 39), thus reducing the risk of metabolic conversion in vivo to metabolites with reduced lipid solubility and greater cross-resistance with CQ.

MATERIALS AND METHODS

Drugs used. AQ and VP were purchased from Sigma, Dorset, United Kingdom. DesAQ and AMOPYRO were obtained from Parke Davis Ltd., Hampshire, United Kingdom. NTBAQ was synthesized as follows.

4-Hydroxy-3-tert-butylaminomethylocetanilide. 4-hydroxycetanilide (1.00 g, 6.6 mmol) was refluxed with tert-butylamine (0.73 g, 10 mmol) and aqueous formaldehyde (1.25 g, 14 mmol; 33% [wt/vol]) in ethanol (50 ml) for 24 h. The solvent was evaporated, and the product was purified by flash column (silica) chromatography using 1 to 5% methanol–dichloromethane as the eluent. The product was obtained as a white solid. 1H nuclear magnetic resonance (CDCl3, 200 MHz) δ 7.25 (1 H, s, NH), 7.20 (1H, d, JNH = 2.20 Hz, Ar-H), 7.02 (1H,
aminoquinoline antimalarial agents are subject to a significant inoculum effect (5, 16). As expected, the measured 50% inhibitory concentration (IC₅₀) of the drug increases as inoculum size increases (where inoculum size = parasitemia × hematocrit) because of drug depletion from the medium. In this study, the drug potency of the compound studied was assessed at inoculum sizes ranging from 1 to 10% (final parasitic volume, 0.0001 to 0.001). Over this range, the relationship between the measured IC₅₀ of the drug and inoculum size is linear. Extrapolation of this line to an inoculum size of zero provides a measure of absolute drug potency based on the following equation: measured IC₅₀ + absolute IC₅₀ = (absolute IC₅₀ × concentration ratio × fractional volume of PRBC), where PRBC stands for parasitized erythrocytes. Furthermore, we have previously validated the use of this mathematical relationship in the determination of the cellular drug accumulation ratio (CAR) based on the following equation (16): CAR = (measured IC₅₀ - absolute IC₅₀)/(absolute IC₅₀ × fractional volume of PRBC), where PRBC stands for parasitized erythrocytes and CAR is the ratio of the amount of drug in the infected cell pellet to the amount of drug in a similar volume of medium. This approach circumvents the need for direct measurement of accumulation using radioactively labelled drug.

**Isolation of peripheral blood leukocytes.** Human polymorphonuclear leukocytes (PMN) were isolated from venous blood of healthy male volunteers (21 to 40 years) by centrifugation onto a density gradient of lymphoprep (4 ml Sigma) and Monoypoly resolving medium (8 ml Sigma). Following centrifugation (1,000 × g, 45 min), the band of PMN was removed from the resolving medium and washed with PBS (pH 7.4). Erythrocyte contamination was eliminated by repeating the resolving procedure. Purified PMN were resuspended in Dulbecco’s PBS (pH 7.4; Sigma). This procedure resulted in a 99% yield of PMN with 95% viability as determined by the Wright staining technique.

**Determination of drug lipophilicity.** Direct cytotoxicity was determined by trypan blue exclusion (35). Isolated PMN (10⁶ cells ml⁻¹) were incubated in Dulbecco’s PBS (Sigma) (37°C; 1 h) in the presence and absence of drug. After incubation, the cells were sedimented and resuspended in drug-free Dulbecco’s PBS. Cell viability was then assessed microscopically by comparing the percentage of test cells able to exclude trypan blue (0.2% [w/v]) with those of control incubations.

**Determination of depletion of intracellular glutathione.** Isolated PMN were suspended in Dulbecco’s PBS, pH 7.4 (37°C; 1 h), at a density of 0.5 × 10⁶ cells ml⁻¹, in the presence and absence of both phosphorol-12'-myristate-13'-acetate (10 ng ml⁻¹) and various concentrations of the drug under study. Glutathione (GSH) levels were determined by the method of Cote and Maldeus (10) as described previously. Bromobimane (3 mM) in N-ethyl morpholine (5 mM; pH 8.0) was added to each incubation mixture, and these incubation mixtures were then left in the dark for 5 min. Protein was precipitated with trichloroacetic acid (100%; 10 μl) and centrifuged (1,000 × g, 5 min). GSH adducts were detected as gradient HPLC as described previously (10) and were detected with a Hitachi 1080 fluorescence detector (excitation wavelength = 304 nm; emission wavelength = 480 nm). The amount of GSH adduct formed was calculated by comparison with standard knowns, Eugenol (4-allyl-2-methoxyphenol), which has been shown to deplete intracellular GSH (58), was used (at concentrations ranging from 1 to 300 μM) as a positive control.

**RESULTS**

As predicted, both AMOPYRO and NTBAQ were more lipophilic than DesAQ at pH 7.2 but were less lipophilic than AQ (Table 1). In agreement with the original hypothesis, the ability of VP to improve antiparasitic potency against CQ-resistant parasites (as shown in Table 1) could be predicted on the basis of this measure of lipophilicity. These data are presented as an activity enhancement index (AEI). This is the ratio of the IC₅₀ of the drug in the absence of VP to the IC₅₀ of the drug in the presence of VP (5 μM). It can be seen that the ability of VP to enhance activity is inversely related to log D at pH 7.2 (Fig. 2). VP can substantially improve the activity of both CQ-resistant and sensitive isolates (AEI = 2.40 at pH 7.2 for the K1 and PH3 isolates, respectively). This effect is markedly reduced with the more lipophilic NTBAQ isolates (AEI = 1.16 and 1.24 for the K1 and PH3 isolates, respectively) and is absent with AMOPYRO and AQ (for which AEI < 1 for both drugs against both the K1 and PH3 isolates [Table 1]).
The absolute IC₅₀s of each drug against each of the four isolates used are displayed in Table 1. Each of these values was calculated from a single inoculum effect experiment involving triplicate drug susceptibility assays at five separate inoculum sizes. In each case, the standard error associated with the slope of the linear fit of the IC₅₀ of the drug versus the inoculum size was always less than 5% and r was greater than 0.96. The absolute drug potency of each of the four drugs against four isolates of *P. falciparum* was found to correlate with the CAR, derived from inoculum effect analysis (Table 1). This observation confirms earlier studies (19). Clearly DesAQ shows the greatest degree of cross-resistance with CQ susceptibility status. Despite having activity comparable to that of AQ against CQ-susceptible isolates, DesAQ is approximately two- to threefold less active than AQ against resistant isolates. In comparison with AQ, AMOPYRO and NTBAQ have superior antimalarial activities against both CQ-resistant and -susceptible isolates, although the degrees of in vitro cross-resistance for all three drugs are similar (Table 1). Importantly, NTBAQ and AMOPYRO, which are less likely than AQ to undergo side chain modification in vivo, show five- to eightfold improvements in activity compared with the in vivo metabolite of AQ, DesAQ, against resistant parasite isolates (Table 1).

Previous studies designed to assess the toxicity of AQ have used PMN in vitro as a marker for toxicity in vivo (37). Figure 3A shows the effects of the compounds studied on the viability of PMN. It can be seen that all four of the compounds studied show little direct toxicity to PMN at concentrations of less than 50 μM. Both NTBAQ and AMOPYRO displayed greater toxicities at concentrations of greater than 50 μM than did AQ and DesAQ. Furthermore, none of the four drugs tested exerted any significant effect on cell recovery at concentrations of less than 50 μM (Fig. 3B).

All four of the drugs studied exhibited similar effects on the level of intracellular GSH in activated PMN (Fig. 4). Indeed, all four compounds depleted intracellular GSH levels at concentrations in excess of 1 μM, with maximal GSH depletion occurring at approximately 100 μM.

**DISCUSSION**

The growing problem of parasite resistance to CQ emphasizes the need to develop novel compounds of this class for the treatment of *P. falciparum*. The 4-aminoquinoline antimalarial agent AQ is highly effective against both CQ-susceptible and -resistant parasites in vitro, although its potency against resistant parasites in vivo is reduced (24, 28, 32). The reduction in AQ’s potency against resistant isolates in vivo is due to the fact that the drug rapidly undergoes first-pass metabolism to DesAQ. This metabolite is less active against CQ-resistant isolates than against the parent drug (4, 6), because DesAQ is a better substrate for the VP-sensitive component of CQ resistance as a result of decreased lipophilicity at pH 7.2 (4).

We have therefore reasoned that a 4-aminoquinoline analog
which has physicochemical properties similar to those of AQ but is metabolically less labile and so cannot be metabolized in vivo to a more water-soluble metabolite would be superior to both CQ and AQ in the treatment of *P. falciparum* malaria in vivo. AMOPYRO and NTBAQ possess these superior structural and physicochemical properties. Both AMOPYRO and NTBAQ show greater levels of lipophilicity than does DesAQ at pH 7.2.

Consistent with previous studies (4, 6), it was found that DesAQ is less active against resistant isolates than is AQ and that VP can selectively increase the activity of DesAQ against these parasites (Table 1). In contrast, the effects of VP on the activity of AMOPYRO and NTBAQ are less pronounced than with the more hydrophilic DesAQ. The activity of AMOPYRO was not influenced by VP, and NTBAQ activity was only slightly increased, by 14 and 19%, against the K1 and PH3 isolates, respectively. These data are consistent with previous findings from this laboratory showing an inverse relationship between lipid solubility characteristics and the VP-sensitive component of resistance (4).

AMOPYRO and NTBAQ show greater levels of activity than both AQ and DesAQ in vitro, although the differences seen with the CQ-susceptible parasite would be of limited importance in a clinical setting. However, against resistant isolates the activities of both AMOPYRO and NTBAQ are clearly superior to that of DesAQ. The observation that the activity of AMOPYRO is similar to that of DesAQ against CO-susceptible isolates but greatly increased compared with that of DesAQ against CQ-resistant isolates in vitro is consistent with earlier reports (1). AQ exhibits an approximately fivefold reduction in activity against CQ-resistant isolates compared with that against CQ-susceptible isolates. This compares to an approximately 10-fold reduction in the potency seen with DesAQ. AQ’s activity, which is enhanced compared with that of DesAQ, is almost certainly due to its reduced affinity for the VP-sensitive resistance mechanism; however, the fact that there is still a fivefold reduction in the activity of AQ against resistant isolates indicates that there could be additional factors involved in the CQ resistance phenotype. Similarly, both NTBAQ and AMOPYRO show an approximately fivefold reduction in activity against resistant isolates; however, these compounds are still between five- and eightfold more potent than DesAQ against these isolates. The differences in activity reported here can be explained in terms of parasite drug accumulation as has been seen with other aminoquinoline drugs (19).

All four drugs in this study exhibited limited toxicity against PMN, which was observed only at high concentrations, i.e., in excess of 50 μM. Several studies have supported the role of oxidative bioactivation to a quinoneimine metabolite as the basis for the side effects, such as hepatotoxicity and agranulocytosis, associated with the prophylactic use of AQ (7, 9, 18). Drug bioactivation to a chemically reactive metabolite can be assessed by monitoring intracellular GSH depletion from activated PMN in vitro (37). Both AMOPYRO and NTBAQ depleted GSH, and therefore it is possible that these compounds also have the potential to form toxic metabolites in vivo. However, previous work from this laboratory has shown that by substitution of fluorine for the hydroxyl group of AQ, it is possible to block the metabolic oxidation of AQ to a quinoneimine, while retaining antimalarial drug potency (26). Such an approach could be of value if a similar toxicity were indeed associated with AMOPYRO and/or NTBAQ. Thus, by the appropriate substitution of both the diethyamine side chain and the hydroxyl moiety, it might be possible to eliminate the problems associated with resistance to and toxicity of AQ in vivo.

In summary, on the basis of an understanding of the pharmacology of both CQ and AQ we have identified two compounds, AMOPYRO and NTBAQ, which have potent antimalarial activity and reduced cross-resistance with CQ. Furthermore, previous studies have indicated that both N-tertbutyl (31) and pyrrolidine (29, 39)-substituted 4-aminoquinolines are metabolically less labile than AQ and are therefore less likely to undergo bioactivation to an N-dealkylated metabolite. These two studies found that the major metabolite of AMOPYRO in humans, namely, the primary amine, accounts for less than 2% of the total dose and that AMOPYRO is excreted essentially unchanged (29, 39). This means that both AMOPYRO and NTBAQ are unlikely to be metabolized to a species that is a substrate for the VP-sensitive component of resistance. Therefore, these pyrrolidine- and N-tertbutyl-substituted compounds should retain antimalarial activity against resistant parasites in vivo.

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REFERENCES