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The Role of Drug Accumulation in 4-Aminoquinoline Antimalarial Potency

THE INFLUENCE OF STRUCTURAL SUBSTITUTION AND PHYSICOCHEMICAL PROPERTIES

Shaun R. Hawley, Patrick G. Bray, Paul M. O'Neill, B. Kevin Park and Stephen A. Ward

DEPARTMENT OF PHARMACOLOGY AND THERAPEUTICS, UNIVERSITY OF LIVERPOOL, LIVERPOOL, L69 3BX, ENGLAND

ABSTRACT. We have investigated a series of novel 4-aminoquinoline analogues related to amodiaquine, that possess side chain modifications designed to influence both drug pKa and lipophilicity. These compounds have been used to determine the influence of physicochemical properties on antimalarial activity against, and accumulation by, both chloroquine-susceptible and chloroquine-resistant isolates of *Plasmodium falciparum*. The compounds tested exhibited a 500-fold range of absolute antimalarial potency. Absolute drug potency and drug accumulation were found to be significantly correlated in each of the four isolates of *Plasmodium falciparum* studied. The level of accumulation was unrelated to lipophilicity and was significantly greater than the predicted levels of accumulation based on drug pKa, compartmental pH, and Henderson-Hasselbach considerations. Further analysis of the relationship between 4-aminoquinoline accumulation and activity implicated the involvement of additional forces in the accumulation process. BIOCHEM PHARMACOL 52;5:723–733, 1996.

KEY WORDS. amodiaquine; 4-aminoquinolines; *Plasmodium falciparum*; accumulation; activity; physicochemical properties

The 4-aminoquinoline series of drugs, in the form of AQ† and CQ, have remained at the forefront of antimalarial chemotherapy, in the treatment of *P. falciparum*, for over fifty years. These compounds are generally well tolerated, cheap, and highly effective in the absence of parasite resistance. It is readily accepted that the ability of these compounds to inhibit parasite growth is dependent upon their ability to accumulate within the parasite to very high concentrations [1]. All of the available evidence suggests that the accumulation of the 4-aminoquinolines occurs predominantly inside the parasite's acid food vacuole [2] and this organelle is, therefore, thought to be the site at which they exert their antimalarial effect [3].

The high levels of accumulation that these compounds are able to achieve within the parasite are thought to result, at least partially, from the fact that they are weak bases [1]. Compounds of this type are able to exist in both charged (protonated) and uncharged (unprotonated) forms. The unprotonated or neutral form of the drug is highly membrane permeable and can diffuse freely and rapidly across biological membranes, whereas the mono- and/or diprotonated forms of the drug are at least an order of magnitude less membrane permeable, and so diffuse across these membranes at a much reduced rate. Therefore, if the unprotonated form of the drug diffuses across a biological membrane into an acidic compartment, it will rapidly become protonated and be unable to diffuse back out again. The concentration of such compounds in acidic compartments is, therefore, predictable and can be determined based on the pH within the compartment, its volume, and the pKa(s) of the drug in question, providing that there are no additional accumulating forces involved [4]. In the case of *P. falciparum*, an effective proton gradient exists between the external environment (pH 7.4) and the acid food vacuole (pH 5.0).

Whether or not the absolute levels of steady-state drug accumulation of the 4-aminoquinolines in *P. falciparum* are due solely to ion trapping, and can, therefore, be predicted simply from their weak base properties is still controversial. Almost all of the studies designed to address this question have used CQ as a substrate. A number of studies have provided evidence in support of simple weak base-driven CQ accumulation [1, 5, 6], whereas other reports have provided contradictory evidence in support of the involvement of additional mechanisms in the accumulation of CQ [7–9].

Corresponding author. Stephen A. Ward, Department of Pharmacology and Therapeutics, University of Liverpool, Liverpool L69 3BX, England. Tel. 0151-794-8219; FAX 0151-794-5540; E-mail: saward@liverpool.ac.uk

† *Abbreviations:* AQ, amodiaquine; CQ, chloroquine; TBQ, tebuquine; 4deOH-AQ, 4′-dehydroxyamodiaquine; 4F-AQ, 4′-dehydroxy-4′-fluoroamodiaqine; 5F-AQ, 5′-fluoroamodiaqine; 5,6diF-AQ, 5′,6′-difluoroamodiaqine; 2,6diF-AQ, 2′,6′-difluoroamodiaquine; 2,5,6triF-AQ, 2′,5′,6′-trifluoroamodiaquine; 4F-TBQ, 4′-dehydroxy-4′-fluorotebuquine; ATBQ, 5′-(4-chlorophenyl)amodiaquine; 4F-ATBQ, 4′-dehydroxy-4′-fluoro-5′-(4-chlorophenyl)amodiaquine; CAR, cellular accumulation ratio; VAR, vacuolar accumulation ratio.

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Studies conducted in this laboratory indicate that the level of accumulation of CQ (at least in CQ-susceptible isolates of *P. falciparum*) can largely be explained in terms of the drugs weak base properties [10]. However, these conclusions do not extend to AQ, for which levels of drug accumulation 2- to 3-fold greater than the substantially more basic CQ were determined. In fact, the experimentally measured extent of AQ accumulation seen in this study was 7-fold greater than could be predicted from the weak base properties of the drug. This enhanced AQ accumulation was also shown to be parasite-specific, because accumulation of both AQ and CQ into a mammalian cell line could be predicted accurately from the drug's weak base properties alone.

To investigate whether or not there is a general relationship between drug accumulation and antiparasitic activity, we have investigated a series of novel 4-aminoquinoline derivatives, based upon side-chain modifications of AQ. These structural analogues were designed to introduce systematic changes to the structural and physicochemical properties (pKas and Log Ds) of the compounds. These compounds have been used to investigate the structural and physiochemical features required for 4-aminoquinoline accumulation and activity and the structural and/or physicochemical basis of the enhanced antimalarial activity of AQ over that of CQ.

MATERIALS AND METHODS

Drugs Used in This Study

The structures of the compounds used in this study are shown in Fig. 1. Amodiaquine (AQ) and tebuquine (TBQ) were obtained from Sigma, Poole, Dorset, U.K. and Parke-Davis, Ann Arbor, MI, respectively. 4deOH-AQ, 4F-AQ, 5F-AQ, 5,6diF-AQ, 2,6diF-AQ, 2,5,6triF-AQ, 4F-TBQ, ATBQ, and 4F-ATBQ were synthesised according to the methods of O'Neill *et al.* [11] and O'Neill *et al.* [12].

Measurement of Drug Dissociation Constants

The dissociation constants of all the compounds used in this study were measured by Sirius Analytical Instruments Ltd (East Sussex, U.K.) using a PCA101 automatic titration apparatus. Due to the poor water solubility of the compounds studied, the pKas of each compound were measured by continuous titration in various methanol/0.15 M KCl ratios, followed by extrapolation to 0% cosolvent by use of the standard Yasuda-Shedlovsky procedure. Experiments were carried out at 25°, under an argon atmosphere to prevent CO_2 ingress. Titrations were carried out with 0.5 M KOH and 0.5 M HCl. The measured pKas refer to 1. the proton reaction involving the terminal side chain diethylamine nitrogen, and 2. the first proton reaction involving the quinoline nucleus (i.e. protonation of the quinoline ring heteroatom nitrogen).

Measurement of Drug Lipophilicity

The relative lipophilicity, at pHs 7.4 and 5.0, of each of the compounds used in this study, was assessed, using an adaptation of the method of Zamora *et al.* [13]. This method involves measuring the partitioning of drug between 1-octanol and 0.1 M PBS (pH 7.4) or 0.1 M acetate buffer (pH 5.0). HPLC grade 1-octanol (Sigma) was presaturated with aqueous phase buffer and, conversely, buffered aqueous phase was presaturated with HPLC grade 1-octanol before use. The drug was dissolved in aqueous phase buffer at a final concentration of 1×10^{-4} M, an equal volume of 1-octanol was added, and the tubes were then continuously inverted for 15 min (experiments carried out over time intervals ranging from 5 to 60 min confirmed that equilibration was reached within 15 min). The final concentration of drug in both aqueous and octanol fractions was assessed by comparing measured levels of UV absorbance (340 nm) of these experimental fractions to those of known drug standards. The partition coefficient, D, was determined by dividing the concentration of drug in 1-octanol by the concentration in the aqueous phase. Log D was used as a measure of lipophilicity.

Parasite Isolates, Maintenance, and Preparation

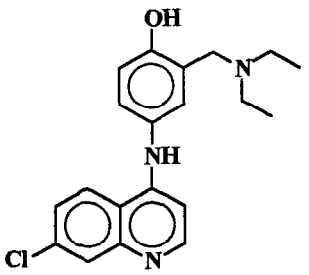
Four isolates of *P. falciparum* were used in this study. The 3D7, HB3, and K1 isolates were kindly provided by Professor D. Walliker, University of Edinburgh, U.K. The PH3 isolate was kindly provided by Professor M. Hommel, Liverpool School of Tropical Medicine, Liverpool, U.K. Cultures were maintained using an adaptation of the methods of Trager and Jensen [14] and Jensen and Trager [15]. Cultures consisted of a 1%–5% suspension of O-positive erythrocytes in complete culture medium (RPMI 1640 medium supplemented with 10% human AB serum, 25 mM HEPES buffer, and 23 mM $NaHCO_3$). These cultures were gassed with an atmosphere of 93% N_2, 3% CO_2, and 4% O_2. Cultures were synchronised by the method of Lambros and Vandenberg [16] 48 h before use.

Drug Sensitivity Assays

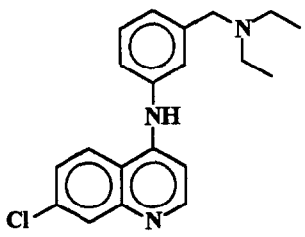
Drug potency was assessed using an adaptation of the standard 48-h microdilution technique described by Desjardins *et al.* [17]. Parasites were exposed to serial dilutions of drug over 48 h and growth was measured by comparing the level of incorporation of [G-^{3}H]hypoxanthine, by the parasites, at each drug concentration, with an appropriate control.

Measurement of Drug Accumulation and Absolute Drug Activity Using Inoculum Effect Analysis

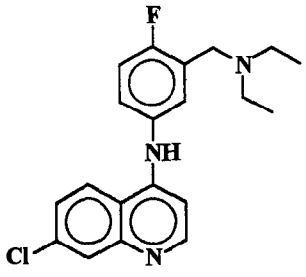
Previous investigations with AQ have indicated a significant 'inoculum effect' with this drug [18]. As a result, measured drug IC_{50} increases with increased inoculum size (where inoculum size = parasitaemia × haematocrit) due to significant drug depletion from the medium. In this study, drug potency of the compounds studied was assessed at inoculum sizes ranging from 1 to 10 (fractional parasite volume 0.0001 to 0.001). Over this range, the relationship



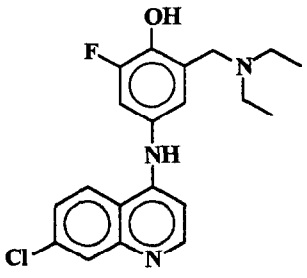
amodiaquine (AQ)



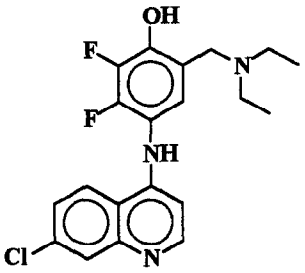
4'-dehydroxyamodiaquine (4deOH-AQ)



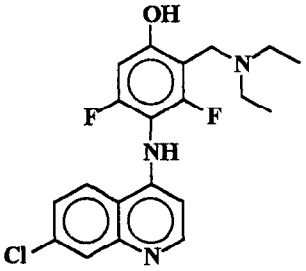
4'-dehydroxy-4'-fluoroamodiaquine (4F-AQ)



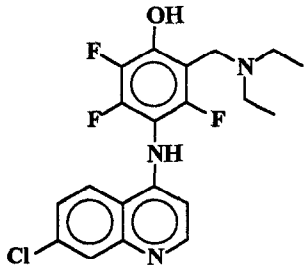
5'-fluoroamodiaquine (5F-AQ)



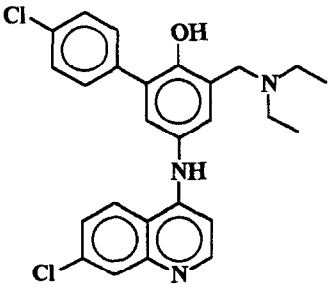
5',6'-difluoroamodiaquine (5,6diF-AQ)



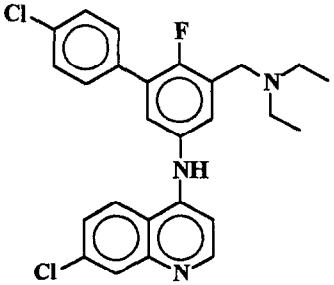
2',6'-difluoroamodiaquine (2,6diF-AQ)



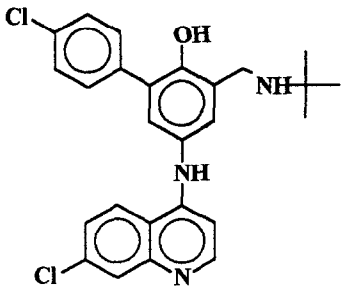
2',5',6'-trifluoroamodiaquine (2,5,6,diF-AQ)



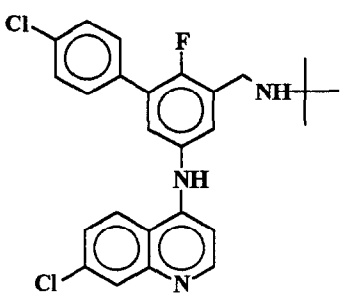
5'-(4-chlorophenyl)amodiaquine (ATBQ)



4'-dehydroxy-4'-fluoro-5'-(4-chlorophenyl)amodiaquine (4F-ATBQ)



tebuquine (TBQ)



4'-dehydroxy-4'-fluorotebuquine (4F-TBQ)

FIG. 1. The chemical structures of the compounds used in this study.

between measured drug IC_{50} and inoculum size is linear. Extrapolation of this line to an inoculum size of zero provides a measure of absolute drug potency from the equation [6]:

$$IC_{50\ measured} = IC_{50\ absolute} + (IC_{50\ absolute} \times \text{accumulation ratio} \times \text{fractional volume of PRBC}) \quad (1)$$

where: PRBC = parasitised red blood cells.

Furthermore, we have previously validated the use of this mathematical relationship for the determination of the cellular drug accumulation ratio from the equation [18]:

$$\text{Accumulation ratio} = \frac{IC_{50\ measured} - IC_{50\ absolute}}{IC_{50\ absolute} \times \text{fractional volume of PRBC}} \quad (2)$$

This approach circumvents the need for direct measurement of accumulation using radioactively labelled drug.

Mathematical Prediction of Drug Accumulation

Cellular accumulation ratios for the drugs studied were predicted from a derivation of the Henderson-Hasselbach equation. Based on a knowledge of drug pKa, and the following assumptions that 1. the proton gradient from the extracellular medium to within the acid vacuole is 2.4 pH units (extracellular pH = 7.4, vacuolar pH = 5.0 [3]; 2. charged (protonated) drugs are membrane impermeable; and 3. there is no intracellular binding of either drug, it is possible to predict compartmental drug distribution using the following equation, as described previously [8]:

$$\frac{[Drug]_v}{[Drug]_o} = \frac{1 + 10^{(pKa\ 1 - pHv)} + 10^{(pKa\ 1 + pKa\ 2 - 2pHv)}}{1 + 10^{(pKa\ 1 - pHo)} + 10^{(pKa\ 1 + pKa\ 2 - 2pHo)}} \quad (3)$$

Where: pHv = pH inside the vacuole (assumed to be pH 5.0 [3])
pHo = pH externally (assumed to be pH 7.4)

$$\frac{[Drug]_v}{[Drug]_o} = \text{Vacuolar drug accumulation ratio (VAR)} \quad (4)$$

Making the assumptions that the parasites acid vacuole occupies 3.2% of its total cell volume [3], corresponding cellular drug accumulation ratios (CARs) for each cell type were then calculated using the following equation:

$$CAR = VAR \times \text{Fractional cell volume occupied by acid vacuoles} \quad (5)$$

RESULTS

Physicochemical Properties

The incorporation of fluorine into drug molecules can affect lipid solubility. Therefore, for this series of compounds, values of drug lipophilicity (Log D) at two different pHs (pH 7.4 and pH 5.0) were measured and are shown in Table 1.

It can be seen that, at pH 7.4, all of the compounds studied were found to be of a similar lipophilicity. All of these compounds are highly lipid soluble at this pH, and the inability to measure differences between individual drug Log Ds at this pH may be a function of this high degree of lipophilicity.

However, at pH 5.0, there is a large difference (more than 1000-fold) between the lipophilicity of the compounds studied. At pH 5.0, the parent molecule, AQ, is essentially diprotonated (Table 1) with a Log D value of −1.4. Replacement of the hydroxyl function with hydrogen has little effect on lipophilicity, whereas isosteric replacement of the hydroxyl function with fluorine increases the Log D (from −1.4 to −0.6) and, hence, lipid solubility. Introduction of fluorine at the 5′-, 5′,6′-, 2′,6′-, and 2′,5′,6′- positions further increases the lipophilicity of the amodiaquine molecule at pH 5.0. It must be noted, though, that AQ and its fluorine-substituted analogues are all essentially hydrophilic at pH 5.0, with the exception of 2,5,6triF-AQ, which is essentially hydrophobic.

TABLE 1. Measured physicochemical properties and protonation equilibria for the compounds studied

	Dissociation constants		Protonation equilibria (at pH 5.0)*		Protonation equilibria (at pH 7.4)*		Log D	
	pKa 1	pKa 2	% Monoprotic	% Diprotic	% Monoprotic	% Diprotic	pH 5.0	pH 7.4
AQ	8.14	7.08	0.753	99.175	52.183	32.432	−1.4	2.61
4deOH-AQ	9.18	7.26	0.540	99.453	56.373	41.995	−1.3	2.44
4F-AQ	8.70	7.26	0.526	99.454	53.367	41.860	−0.6	2.19
5F-AQ	7.81	6.24	5.280	94.565	65.680	6.542	−0.5	2.13
5,6diF-AQ	7.50	5.44	26.352	73.333	54.664	1.088	−0.2	2.14
2,6diF-AQ	7.94	6.21	5.692	94.193	71.526	6.103	−0.3	2.53
2,5,6triF-AQ	7.49	4.64	69.121	30.556	54.987	0.170	0.28	1.99
TBQ	7.84	6.75	1.603	98.253	55.062	18.301	1.00	2.70
4F-TBQ	8.96	6.90	1.232	98.757	73.296	24.024	1.22	2.69
ATBQ	7.37	6.20	5.51	94.065	42.332	5.935	1.93	2.64
4F-ATBQ	8.69	6.81	1.505	98.475	74.675	20.446	1.84	2.70

* Calculated from the Henderson-Hasselbach equation.

The pKas of CQ, the most widely used 4-aminoquinoline antimalarial, are reported to be 10.2 (pKa 1) and 8.1 (pKa 2) [19]. To rationalise the effects of the chemical substitutions performed in this study on the dissociation constants of the compounds in question, it is simplest to consider the stepwise chemical alterations from CQ. Introduction of a benzene ring into the side chain of CQ (producing 4deOH-AQ) decreases both of the pKas of interest (pKa 1 is reduced from 10.2 to 9.18, pKa 2 is reduced from 8.1 to 7.26). This reduction in basicity is due to the presence of the aromatic ring, which serves to accept electrons from the 4′-position amino function and from the diethylamino side-chain function. Introduction of a 4′-position hydroxyl function into the aromatic functionality of 4deOH-AQ further decreases pKa 1 (from 9.18 to 8.14), having little effect of pKa 2 (7.26 versus 7.08). This reduction in the basicity of the side chain is most likely a result of a hydrogen-bonding interaction between the hydroxyl function proton and the side-chain diethylamino nitrogen (Fig. 2). Such an interaction occupies the electron lone pair on the diethylamino nitrogen, therefore, reducing its basicity.

The effects of fluorine substitution on the pKa of the terminal diethylamine nitrogen (pKa 1) can also be rationalised by taking into account this hydrogen-bonding interaction of the hydroxyl proton with the tertiary diethylamine nitrogen atom (Fig. 2). Replacement of the hydroxyl function of AQ with fluorine raises the pKa of the side-chain nitrogen (from 8.14 to 8.70), in the same way as replacement of the hydroxyl function of AQ with hydrogen does. This increase in basicity is due to the fact that, in the absence of the 4′-position hydroxyl function, the electron lone pair on the diethylamino nitrogen is no longer involved in an intramolecular hydrogen bond. Fluorine substitution adjacent to the hydroxyl group at the 5′-position (e.g., 5F-AQ, 5,6diF-AQ, and 2,5,6triF-AQ) lowers the pKa of the side-chain diethylamino nitrogen atom. This effect is almost certainly as a consequence of the effect of fluorine substitution on the acidity of the 4′-hydroxyl function. A fluorine atom at the 5′-position weakens the hydroxyl-hydrogen bond with a resultant increase in hydrogen bonding with the side-chain nitrogen. As a result, protonation of the diethylamino functionality becomes more difficult, with a resultant decrease in pKa.

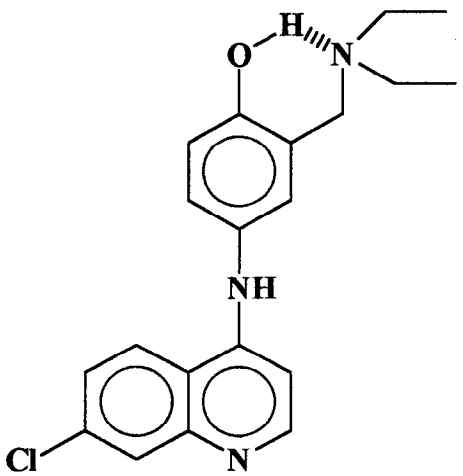


FIG. 2. Proposed hydrogen bonding interaction between the 4′-position hydroxyl function and the diethylamine side chain of amodiaquine.

The general effect of fluorination on the pKa of the aminoquinoline functionality (pKa 2) is a decrease in basicity. This is most pronounced for 2,5,6triF-AQ, which is approximately 2.5 pKa units less basic than AQ. The effects of fluorine on the basicity of the 4-aminoquinoline functionality can be rationalised by the strong electron withdrawing effects of fluorine destablising the positively protonated 4-aminoquinoline functionality.

The introduction of a 4-chlorophenyl group into the 5′-position of AQ produces a large increase in lipophilicity at pH 5.0 (Log D, pH 5.0, for AQ = −1.4, and for ATBQ Log D, pH 5.0, =1.0), but appears to have little effect on lipophilicity at pH 7.2 (this may, again, be a result of insufficient sensitivity at pH 7.2 as discussed earlier). The introduction of a 4-chlorophenyl function into this position has a similar effect on the pKa of the side chain (lowering pKa 1 from 8.14 to 7.37), as that seen with the introduction of fluorine in the same position. This phenomenon is more difficult to rationalise but may be due to enhanced intramolecular hydrogen-bonding interactions between the 4′-position hydroxyl group and the side-chain nitrogen. Conversion of the tertiary nitrogen atom in ATBQ to a secondary amine (producing TBQ) increases the basicity of the side chain, as expected (increasing pKa 1 from 7.37 to 7.84). Replacement of the hydroxyl function in TBQ and ATBQ with fluorine raises the pKa of the side chain (by approximately 1.2 pKa units in each case) as in the AQ series. This can be rationalised, once again, by the loss of hydrogen bonding with the side chain following substitution of the hydroxyl function for fluorine (Fig. 2).

It can be seen from Table 1 that perturbations in the pKas, across the AQ analogue series, lead to differences in protonation equilibria at both pH 5.0 and 7.4. These differences in equilibria, at least at pH 5.0, are also reflected in the measured lipophilicities. At pH 5.0, the most highly protonated compounds, such as AQ and 4F-AQ, are as expected, those compounds that are the most hydrophilic. Conversely, those drugs least protonated are also the most highly hydrophobic. Interestingly, although there are distinct differences between the protonation equilibria of the AQ analogues at pH 7.4, there is little difference between the lipophilicities of these compounds, which are all highly lipophilic, at this pH. Once again, this may be a result of an inability to discriminate between small differences in individual drug Log Ds at this pH due to this high level of lipophilicity.

Biological Properties

A representative example of the effect of increasing inoculum size on drug sensitivity is shown in Fig. 3a, indicating

a greater than 3-fold increase in apparent drug IC_{50}, against the 3D7 isolate, as inoculum size is increased from 1 to 10. Figure 3b confirms the linear relationship that exists between drug IC_{50} and the corresponding inoculum size. The relationship between activity and inoculum size for all of the compounds tested, and all four of the parasite isolates used, remained linear throughout the range of inoculum sizes used ($r > 0.96$ in each case).

The data presented in Table 2 shows the individual absolute drug IC_{50}s, calculated at an inoculum size of zero using the inoculum effect experiment, for each of the compounds, against each of the four isolates of *P. falciparum* studied. The absolute drug IC_{50}s, for each isolate displayed in this table, were calculated from a single inoculum effect experiment involving triplicate drug sensitivity assays at 5 separate inoculum sizes. In each case, the standard error associated with the slope of the linear fit of drug IC_{50} vs inoculum size was always less than 5% and $r > 0.96$. Also shown in Table 2 (in parentheses) are experimentally determined drug IC_{50}s at an inoculum size of 2.5. It can be seen that, at an inoculum size of zero, there is a greater than 500-fold difference in the IC_{50} responses of the compounds tested. The IC_{50} values obtained for AQ were 2.4, 3.7, 16.8, and 10.5 nM against the 3D7, HB3, K1, and PH3 isolates, respectively. It is clear that CQ-resistant isolates K1 and PH3 are less susceptible to the actions of this compound than the CQ-susceptible isolates 3D7 and HB3.

Removal of the 4′-position hydroxyl group (to produce 4deOH-AQ) led to reduction in activity against susceptible isolates without having a significant effect on potency against resistant isolates. The introduction of one fluorine (as in the case of 4F-AQ and 5F-AQ) into the para-hydroxyanilino moiety of AQ produced reduced levels of drug potency in comparison to the parent compound (AQ). The reduction in the level of drug potency of these mono-substituted compounds, in comparison to AQ, was approximately 7-fold and 2-fold for 4F-AQ, against the CQ-susceptible and CQ-resistant isolates, respectively; and 12-fold and 4-fold for 5F-AQ, against the CQ-susceptible and CQ-resistant isolates, respectively. It can be seen that, in each case, this loss of activity was more pronounced in the CQ-susceptible isolates, so that there no longer appeared to be any real differences in the potency of these compounds against CQ-susceptible or CQ-resistant isolates.

The introduction of two fluorines into the para-hydroxyanilino moiety of AQ (to give 5,6diF-AQ and 2,6diF-AQ) produced similar reductions in drug activity, compared to the parent compound, as those seen with the mono fluoro substitutions. Once again, these disubstitutions produced a more marked reduction in the activity of these compounds against CQ-susceptible isolates than against CQ-resistant isolates, resulting in similar IC_{50}s, for both compounds, against all isolates.

The introduction of three fluorine atoms into the para-hydroxyanilino moiety of AQ, producing 2,5,6triF-AQ, resulted in the most substantial loss of antimalarial potency of all the substitutions that were made. Indeed, the introduction of this third fluorine atom produced a compound that was between 35- and 200-fold less active than AQ, and approximately 20-fold less active than the mono- and di-fluorinated compounds.

The introduction of a 4-chlorophenyl group, ortho, to the phenolic hydroxyl moiety of AQ, producing ATBQ, led to a loss of activity against the CQ-susceptible isolates, 3D7 and HB3, although drug potency against the CQ-resistant isolates, in comparison to AQ, was retained. Replacement of the diethylamine side chain of ATBQ with an N-tertbutyl group, to give a pharachlorophenyl and N-tertbutyl substituted analogue of AQ, namely TBQ, produced a compound that was between 2.5- and 13-fold more active than ATBQ, and between 2- and 4-fold more active than AQ. Indeed, TBQ was found to be the most potent of all of the compounds tested, with IC_{50}s (nM) of 1.22, 0.9, 4.3, and 2.8 against the 3D7, HB3, K1, and PH3 isolates, respectively.

It would appear that the compounds showing the greatest

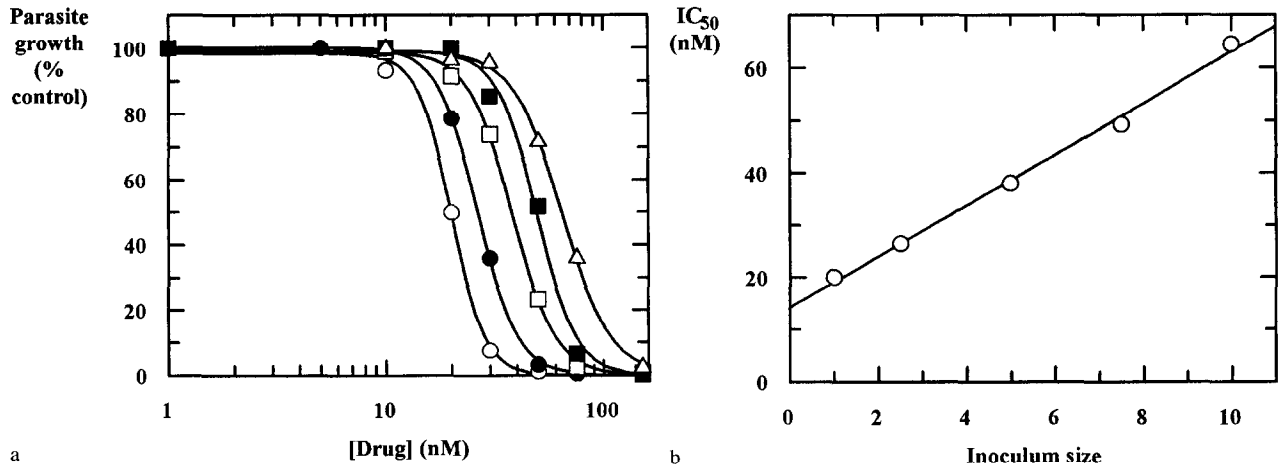


FIG. 3. A representative example (using the 3D7 isolate) of the effect of increasing inoculum size (parasitaemia × haematocrit) on drug (ATBQ) potency (a) ○, inoculum size of 1; ●, inoculum size of 2.5; □, inoculum size of 5; ■, inoculum size of 7.5; △, inoculum size of 10), and the corresponding graph of measured IC_{50} versus inoculum size (b).

TABLE 2. Drug potency at an inoculum size of zero, calculated from inoculum effect analysis*

	Experimentally derived absolute drug IC_{50} (nM)			
	3D7	HB3	K1	PH3
AQ	2.4 (8.1)	3.7 (15.8)	16.8 (37.2)	10.5 (23.9)
4deOH-AQ	14.3 (31.1)	16.2 (29.8)	14.7 (57.6)	19.3 (48.5)
4F-AQ	14.3 (23.8)	31.9 (49.5)	38.8 (60.8)	15.2 (23.6)
5F-AQ	21.4 (56.4)	55.2 (75.6)	54.2 (105.4)	49.3 (69.7)
5,6diF-AQ	20.6 (37.7)	32.6 (40.4)	33.7 (54.3)	35.9 (51.0)
2,6diF-AQ	14.5 (25.2)	46.9 (55.3)	24.2 (42.8)	31.4 (50.5)
2,5,6,triF-AQ	490.6 (528.3)	461.9 (536.1)	592.5 (676.3)	653.5 (679.7)
TBQ	1.22 (17.3)	0.9 (8.5)	4.3 (20.8)	2.8 (7.4)
4F-TBQ	49.4 (58.6)	61.1 (81.4)	65.4 (74.3)	54.6 (66.4)
ATBQ	14.3 (26.4)	12.1 (32.7)	18.4 (42.1)	7.1 (13.6)
4F-ATBQ	62.5 (83.9)	72.1 (98.2)	91.1 (101.3)	87.1 (96.4)

* Values in parentheses are IC_{50} values obtained experimentally at an inoculum size of 2.5.

levels of activity are also those that show the greatest levels of cross-resistance between the CQ-susceptible and CQ-resistant isolates. It must also be noted that when the 4′-position hydroxyl group of AQ, TBQ, and ATBQ is substituted by a fluorine atom, there is a significant reduction in antimalarial potency in each case.

Although the relationship between drug activity in each isolate was not absolute, the general rank order of activities was: TBQ ≫ AQ > ATBQ = 4deOH-AQ > 4F-AQ > 2,6diF-AQ = 5,6diF-AQ = 5F-AQ > 4F-TBQ > 4F-ATBQ ≫> 2,5,6triF-AQ.

Table 3 shows experimentally derived cellular drug accumulation ratios, at external IC_{50} concentrations, for each of the compounds tested, against each of the four isolates of *P. falciparum* studied. Cellular accumulation ratios were derived, as described in methods, from the graphs of drug IC_{50} vs inoculum size. The compound with the highest cellular accumulation ratio across all of the four isolates of *P. falciparum* studied was TBQ, which was also the most active compound, and the compound accumulated least by all of the four isolates was 2,5,6tri-F-AQ, the least active compound. The derived cellular accumulation ratios for TBQ, across the four isolates studied, ranged from 6469 in the PH3 isolate, to 79,456 in the 3D7 isolate, and the derived cellular accumulation ratios for 2,5,6tri-F-AQ, across the four isolates, ranged from 179 in the PH3 isolate to 651 in the HB3 isolate. In comparison, the experimentally derived cellular accumulation ratios for AQ were 3653 and 4824, in the K1 and PH3 isolates, and 8955 and 12,613 in the 3D7 and HB3 isolates.

TABLE 3. Experimentally derived cellular accumulation ratios (CARs)

	Experimentally derived CARs*				Predicted CARs
	3D7	HB3	K1	PH3	
AQ	8955	12613	3653	4824	587
4deOH-AQ	3714	3089	5492	1647	845
4F-AQ	2441	1711	1878	2101	829
5F-AQ	3941	1514	3614	1474	101
5,6diF-AQ	2735	923	2348	1590	17
2,6diF-AQ	3068	928	3202	2226	102
2,5,6triF-AQ	298	651	455	179	6
TBQ	79456	70156	14584	6469	290
4F-TBQ	905	1081	1495	522	481
ATBQ	3409	6957	4832	3791	63
4F-ATBQ	928	1290	1158	539	403

* Derived from the inoculum effect analysis and predicted cellular accumulation ratios calculated using the Henderson-Hasselbach equation.

The clear relationship between drug IC_{50} and experimentally derived levels of drug accumulation are illustrated in Fig. 4a, b, c and d, which show correlations of log drug IC_{50} (at an inoculum size of zero) vs log-derived drug accumulation ratios for the 3D7 ($r = 0.948$, $P < 0.0001$), HB3 ($r = 0.935$, $P < 0.0001$), K1 ($r = 0.955$, $P < 0.0001$) and PH3 ($r = 0.953$, $P < 0.0001$) isolates of *P. falciparum*, respectively.

Mathematically predicted cellular accumulation ratios for each drug are also shown in Table 3. These values are predicted from the corresponding measured pKa values and the Henderson-Hasselbach equation by the methods described in Materials and Methods. It must be noted that, for all of the compounds tested, the experimentally derived cellular accumulation ratio was always greater than the mathematically predicted level. Indeed, the experimentally derived levels of drug accumulation were between 2- and 300-fold greater than could be predicted by the drug's weak base properties across the four isolates studied.

It is important to note that in all of the four isolates studied, no significant correlation was found between 1. experimentally derived cellular accumulation ratio and mathematically predicted cellular accumulation ratio ($r < 0.06$, $P > 0.86$); 2. absolute drug IC_{50} and Log D at pH 7.4 ($r < 0.55$, $P > 0.08$); 3. absolute drug IC_{50} and Log D at pH 5.0 ($r < 0.10$, $P > 0.77$); 4. experimentally derived drug accumulation ratio and Log D at pH 7.4 ($r < 0.45$, $P > 0.17$); 5. experimentally derived drug accumulation ratio and Log D at pH 5.0 ($r < 0.19$, $P > 0.58$); 6. Log absolute drug IC_{50} and Log D at pH 7.4 ($r < 0.56$, $P > 0.07$); 7. Log

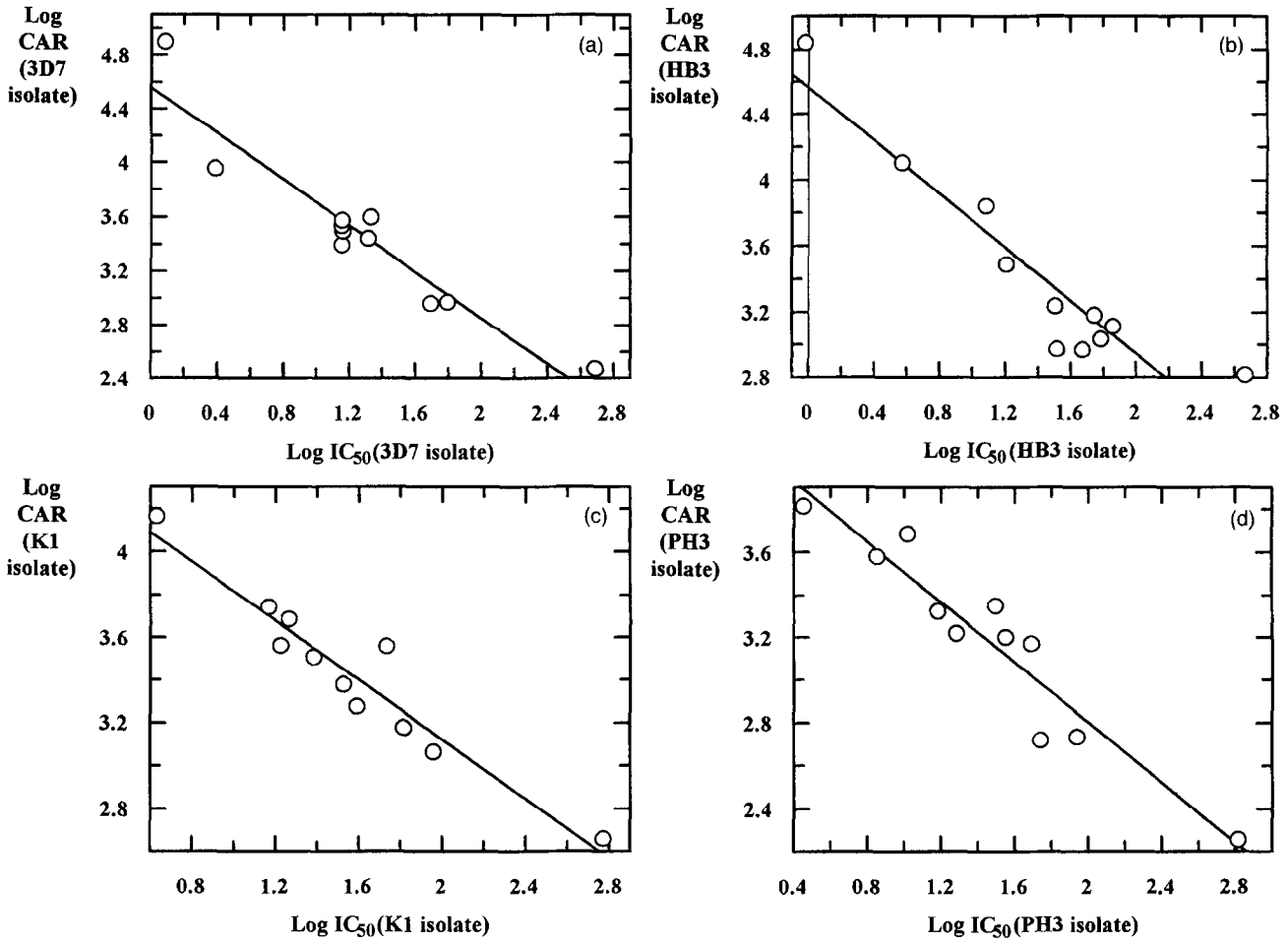


FIG. 4. Correlations of Log-derived cellular accumulation ratio and Log absolute IC_{50} (calculated from inoculum-effect analysis) for the (a) 3D7, $r = 0.948$, $P < 0.0001$, (b) HB3, $r = 0.935$, $P < 0.0001$; (c) K1, $r = 0.955$, $P < 0.0001$; and (d) PH3, $r = 0.953$, $P < 0.0001$ isolates.

absolute drug IC_{50} and Log D at pH 5.0 ($r < 0.24$, $P > 0.47$); 8. Log experimentally derived drug accumulation ratio and Log D at pH 7.4 ($r < 0.53$, $P > 0.09$); and 9. Log experimentally derived drug accumulation ratio and Log D at pH 5.0 ($r < 0.21$, $P > 0.53$).

DISCUSSION

Previous studies [20–23] have shown that AQ possesses greater antimalarial potency than CQ against both CQ-resistant and -susceptible isolates of *P. falciparum*. The enhanced potency of AQ can be explained in terms of differences in the levels to which the two drugs accumulate within the malaria parasite [10]. The differences in the levels of CQ and AQ accumulation cannot be explained solely in terms of the weak base properties of the two drugs. Indeed, AQ is a weaker base than CQ and would, therefore, be expected to accumulate in lower concentrations if the only driving force for drug accumulation was the parasite's transmembrane proton gradient, according to the Henderson-Hasselbach model of weak base accumulation. In the present study, using a larger series of compounds with varying physicochemical properties, this relationship between drug potency and the level of drug accumulation was found to hold in four separate isolates of *P. falciparum*. Critical analysis of all the earlier data suggest that, although physicochemical characteristics are of general importance to aminoquinoline activity, additional structural considerations are necessary to explain the absolute activity patterns of individual compounds within the group. In the present study, no simple relationship was found between either potency or accumulation and lipophilicity as measured by Log D at pH 5.0 and 7.4 or with drug pKa.

The only structural difference between AQ and CQ is the presence of a phenolic group in the side chain of AQ compared to the simple aliphatic side chain in CQ. The presence of this phenolic moiety in AQ changes the chemical characteristics of the compound from that of CQ in a number of ways. First, the introduction of the aromatic benzene ring serves to increase the overall lipophilicity of the compound and decrease the compound's basicity because it is an electron-withdrawing group. Second, the introduction of the hydroxyl function provides a group that can participate in both inter- and intramolecular interac-

tions. These intramolecular interactions may, in turn, affect the physicochemical properties of the compound.

We have taken a simple approach based on stepwise structural modification to determine the relative importance of these structural features in drug accumulation and activity. Introduction of the benzene ring into the side chain of CQ, producing 4deOH-AQ, reduced both pKa 1 and pKa 2 compared to CQ. This is, as expected, due to the electron-withdrawing effect of the benzene ring. This reduction in basicity was associated with increased drug potency, in comparison to previously published activity data for CQ from within our own laboratory [24]. This increased potency was selective against the CQ-resistant isolates. This effect is in keeping with the observations of Bray *et al.* [25] who showed that relative differences in activity of a drug between CQ-susceptible and -resistant isolates, decreased logarithmically as drug lipophilicity at pH 7.2 increased.

Despite these changes, the introduction of the single aromatic ring could not account for all of the enhanced drug accumulation seen with AQ (Table 3). The presence of a 4′-position hydroxyl group (AQ) leads to significantly increased activity and accumulation of the compound in the susceptible isolates, but having little effect on activity against resistant isolates. This additional substitution of a 4′-position hydroxyl group for hydrogen (to give AQ) further decreases the basicity of the compound, making little change to the drug's lipophilicity, in comparison to 4deOH-AQ. The fact that 4deOH-AQ is more basic than AQ and that both compounds have comparable lipophilicity and activity against resistant isolates suggest that the enhanced accumulation and activity of AQ in comparison to 4deOH-AQ, against susceptible isolates, must be a function of the hydroxyl group and its structural characteristics, rather than an effect on physicochemical drug properties.

Fluorine substitution around the phenolic group of AQ has been used to further examine the importance of chemical structure and physicochemical characteristics. The reason for choosing fluorine substitution was that first, fluorine is very similar in size to hydrogen and substitution of fluorine for hydrogen will cause little if any steric changes; second, the fluorine-carbon bond possesses a strong dipole moment that can interact with other dipoles and, as such, can partially mimic the hydrogen bonding effects of the hydroxyl group; and third, fluorine is the most electronegative element known and when substituted for hydrogen is able to alter, quite drastically, such parameters as neighbouring group stability and reactivity, dipole moments and, most importantly, pKa. Therefore, the presence of fluorine in the aromatic ring can selectively alter the physicochemistry of a molecule without any major effect on the 3-dimensional structure [26].

Introduction of one or two fluorines into the phenolic moiety of AQ results in a significant decrease in drug potency in the CQ-susceptible isolates, but has no real effect on potency against the CQ-resistant isolates, in comparison to AQ. Indeed, the enhanced potency seen with AQ against susceptible isolates is lost upon replacement of the phenolic group with fluorine. This, again, indicates the importance of the hydroxyl group in the enhanced activity seen against susceptible parasite isolates. Further, the reduced potency of the analogues fluorinated at positions other than the 4′-position suggests that these substitutions interact negatively with the hydroxyl group, abolishing this selective enhancement of activity and accumulation.

Previous reports suggest that TBQ possesses greater antimalarial activity compared to amodiaquine *in vivo* in experimental animals [27, 28]. These findings are supported by our *in vitro* observations. TBQ was found to be approximately 3-fold more active against susceptible isolates and 4-fold more active against resistant isolates, compared to AQ. This enhancement of activity in both sets of isolates must be a result of either the introduction of a 4-chlorophenyl function or the substitution of a N-tertbutyl side chain for the diethylamino side chain. These substitutions will, obviously, alter both the structural and physicochemical properties of the compound. It is possible that the increased lipid solubility of these 4-chlorophenyl substituted compounds may allow significant transmembrane movement of protonated drug. This will negate condition 3. in our original model of predicted drug accumulation and, as such, it may be more appropriate to consider these four compounds in isolation. Introduction of the 4-chlorophenyl group into AQ (ATBQ) resulted in a reduction in activity selectively against the susceptible isolates. Therefore, it would appear that the 4-chlorophenyl function alone is not responsible for the enhanced activity of TBQ, which must, therefore, be either a result of the secondary amine side chain (an N-tertbutyl side chain) or a combination of both N-tertbutyl side chain and the presence of the 4-chlorophenyl function.

The importance of the 4′-position hydroxyl is emphasised further by 4′-position fluorination of the analogues containing a 4-chlorophenyl function, namely ATBQ and TBQ. In both cases, the replacement of the 4′-position hydroxyl group with fluorine resulted in significant loss of antiparasitic activity and accumulation.

Previous studies have failed to highlight a relationship between the level of drug accumulation and drug potency [1, 29]. However, the data presented here, based on this series of closely related antimalarials, confirms that there is an intimate relationship between the absolute level of antiparasitic drug activity and the level of drug accumulation. We have previously proven the validity of the inoculum effect experiment as a mathematical approach to deriving levels of drug accumulation, by comparison with actual measured levels of accumulation of radiolabelled compound [18]. The observation that levels of drug accumulation are correlated closely with levels of drug potency is striking. The correlation between these two parameters, for 10 compounds in 4 separate isolates, was significant and always greater than $r = 0.93$ ($P < 0.0001$).

The actual levels of drug accumulation observed in this study are always greater than could be predicted by the drug's weak base properties alone. We have recently reported similar findings for AQ in CQ-susceptible isolates [10]. The conclusion of this earlier work was that an additional pH-dependent binding component was responsible for most of the accumulation of AQ. The data presented here are in support of this argument. Clearly, there is no need to invoke differences in target site specificity to explain the sensitivity differences seen, unless the site of drug action is also the site of selective accumulation.

It is also interesting to note that the measured levels of drug accumulation of the nonfluorinated, 4′-position hydroxylated compounds, AQ, TBQ, and ATBQ, are on average approximately 12-, 170-, and 100-fold greater than can be predicted, respectively. In contrast, when the 4′-position hydroxyl group of these compounds is replaced with fluorine, the measured values of accumulation for 4F-AQ, 4F-TBQ, and 4F-ATBQ are, on average, approximately only 2-, 2-, and 3-fold greater than can be predicted, respectively. Furthermore, substitution of the 4′-position hydroxyl group of AQ with hydrogen, producing 4deOH-AQ, also lowers the measured level of accumulation, so that it is only 4-fold greater than can be predicted (differences of between 2- and 4-fold between predicted and mathematically derived cellular accumulation ratios are within the errors associated with the assumptions of our model). These data further strengthen the argument that the presence of the 4′-position hydroxyl group is important in the attainment of enhanced drug accumulation and, subsequently, activity for the compounds in question.

Data presented here, indicating that the measured level of AQ accumulation, and that of a number of closely related drugs, is always greater than can be predicted based solely on the weak base characteristics of the drug, is consistent with earlier findings [10]. This difference, however, may not be significant in CQ-resistant isolates. In this earlier study, evidence was presented to indicate that this enhancement in accumulation may be a consequence of specific intraparasitic drug binding, possibly to ferriprotoporphyrin IX. It is reasonable, therefore, to extend this thinking to the mechanism of accumulation of the compounds studied here. It is also reasonable to assume that the differences in levels of accumulation, seen upon structural modification of AQ, are a consequence of alterations in drug binding. With these assumptions in mind, it would appear that the presence of the 4′-position hydroxyl group in such compounds provides the compound with an enhanced ability to bind. Introduction of a N-tertbutyl side chain or a 4-chlorophenyl group may also enhance drug binding, resulting in the increased activity seen with TBQ and ATBQ. It is important to note that the enhancement of drug activity and accumulation seen in the presence of a hydroxyl group (unhindered by the presence of other fluorine substitutions) or in the presence of a combination of both an N-tertbutyl side chain and a 4-chlorophenyl function is unlikely to be due to an alteration in the physicochemical properties of the drug, because no direct correlations were achieved between either activity or accumulation and any other physicochemical parameter. The observation that internal drug concentration at IC_{50} (calculated from experimentally derived CAR × absolute drug IC_{50}) for 2,5,6triF-AQ are always higher than those of the other compounds studied, could be due to relative high Log D and the high percentage of monoprotic drug at pH 5.0. This would suggest that the interaction with the putative intraparasitic receptor involves diprotonated drug.

As mentioned earlier, there is a direct relationship between the level of drug accumulation and drug potency. However, this relationship is a double exponential and not a linear relationship. Although the interpretation of this would be that drugs could be designed with increased activity if accumulation were increased, the Log-Log relationship means that progressive enhancement of drug potency will require disproportionately larger increases in drug accumulation to be achieved. We have previously attempted to rationalise these observations based upon the accepted drug uptake characteristics of CQ and AQ. Both drugs exhibit biphasic accumulation characteristics [10, 30, 31] comprising a high-affinity saturable component that we argue is parasite-specific and pharmacologically important, and a low-affinity nonsaturable component that is not parasite-specific and may be of toxicological relevance. Based upon this model, the ideal aminoquinoline would be one that would selectively saturate the high-affinity component at drug concentrations at which the amount of drug at the low-affinity site was negligible. Increases in global drug accumulation would lead to an increase in the relative importance of the low-affinity component. It is, therefore, vital in the development of new antimalarial compounds to identify structural and/or physicochemical features of such compounds that greatly enhance the drug's interaction with this high-affinity, low-capacity component, and reducing the compound's interaction with the low-affinity, high-capacity component.

In conclusion, we have shown that, for a series of active 4-aminoquinoline antimalarials, there is a relationship between the logarithm of the mathematically derived cellular drug accumulation ratio and the logarithm of the absolute drug IC_{50} determined at an inoculum size of zero. There is no simple relationship between drug accumulation and either pKa or lipophilicity. We have, however, identified a number of structural features that enhance both drug accumulation and antiparasitic activity and, therefore, suggest the presence of a specific aminoquinoline binding site(s) within the parasite. Identification and characterisation of such a binding site(s) will facilitate the synthesis of safer and more effective antimalarials.

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