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Structure-Activity Relationships and Modes of Action of 9-Anilinoacridines against Chloroquine-Resistant *Plasmodium falciparum* In Vitro

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An in vitro investigation of the structure-activity profiles for a range of 9-anilinoacridines on drug-resistant *Plasmodium falciparum* is reported. C-3, 6-diamino substitution, low lipophilicity, and high pKa values substantially increased the activities of the 9-anilinoacridines tested. There appeared to be no correlation between DNA binding and antimalarial activity. 3,6-Diamino-1′-amino-9-anilinoacridine (compound 13) was the most active compound tested; it had a 50% inhibitory concentration of 25 nM. In vitro mammalian cell growth assays showed compound 13 to be one of the least cytotoxic 9-anilinoacridines (50% inhibitory concentration, 15 μM). Both compound 13 and the antimalarial drug pyronaridine inhibited the decatenation activity of *P. falciparum* DNA topoisomerase II at concentrations of 10 and 11 μM, respectively.

The incidence of drug resistance in patients with *Plasmodium falciparum* malaria is continuing to increase in large areas of the world and is a substantial public health problem (14). This issue has prompted the active and rational development of alternative drugs that may be effective against such drug-resistant strains.

Recently, we reported that several 1′-substituted 9-anilinoacridines, and particularly 1′-NH-alkyl derivatives, show promising in vitro activity against chloroquine-resistant *P. falciparum* isolates (6). Here we report the results for a wider series of 9-anilinoacridines; we studied structure-activity relationships for both in vitro antimalarial activity and mammalian cell toxicity. A comparison of the structure-activity relationships among the various substituted 9-anilinoacridines indicated modifications which substantially increase the in vitro activity against *P. falciparum*. To evaluate the effects of the same 9-anilinoacridines on mammalian cells, the 50% inhibitory concentrations (IC_{50}s) of the compounds against a mammalian (leukemia) cell line were determined. Many 9-anilinoacridines (e.g., amsacrine) are potent inhibitors of mammalian DNA topoisomerase II (13), and we have confirmed that the 9-anilinoacridine derivative most active against *P. falciparum* inhibits the type II topoisomerase in partially purified preparations from *P. falciparum*. Pyronaridine, a 9-anilino-aza-acridine which is showing promise in clinical trials against drug-resistant human malaria in the People's Republic of China (4, 8), also inhibited *P. falciparum* topoisomerase II.

These results and those of previous studies (7) showing that 9-anilinoacridines can be modified to selectively target topoisomerase II in drug-resistant mammalian cells suggest that tailoring such compounds to specifically inhibit the malarial parasite topoisomerase II may be a feasible approach for treating drug-resistant malaria.

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MATERIALS AND METHODS

The new 9-anilinoacridines described here were prepared by slight modifications of previously published methods (5). Full details of the synthesis will be published elsewhere. Pyronaridine was obtained from the Walter Reed Army Hospital, Washington, D.C. Kinetoplast DNA was purchased from Topogen.

Drug lipophilicity was measured by thin-layer chromatography on a cellulose support at a pH of ca. 2 as reported previously (3). The relative mobility (R_m) [log $(1/R_f - 1)$]) values of many 9-anilinoacridines determined in this way have correlated well with log P (n-octanol–water partition coefficient) values (2). However, the method does have drawbacks with polybasic compounds of the type considered here, since relationships between the charge state of the compounds in the chromatography buffer and under physiological conditions are not clear. Ionization constant (pKa) values for both the acridine ring and (where applicable) the side chains were obtained from the literature where possible and were estimated by using data for related compounds when information in the literature was not available (5).

The activities of the drugs (IC_{50}) against chloroquine- and pyrimethamine-resistant *P. falciparum* K1 were measured by incubating *P. falciparum*-infected erythrocyte suspensions (0.5% initial parasitemia) with drugs for 24 h at 37°C. [^{3}H]hypoxanthine (0.25 μCi, 6.2 Ci/mmol) was then added to each sample and cultures were incubated for an additional 24 h. Drug activity was recorded as the concentration of drugs required to inhibit the incorporation of [^{3}H]hypoxanthine into parasites by 50% compared with that required for untreated controls (6). The inhibitory activities of the drugs against *P. falciparum* topoisomerase II were assayed by decatenation of kinetoplast DNA essentially by the method of Riou et al. (11). Briefly, extracts of *P. falciparum* DNA topoisomerase II were incubated with kinetoplast DNA networks at 37°C for 30 min in the presence of Mg^{2+} and ATP. Reactions were stopped by the addition of sodium dodecyl sulfate, and the products were analyzed by agarose gel electrophoresis. Inhibition of *P. falciparum* topoisom-

erase II decatenating activity was measured by adding suitable concentrations of each drug to the standard assay. Extracts of *P. falciparum* with type II topoisomerase activity were prepared from parasite pellets recovered by centrifugation after saponin lysis of infected erythrocytes. The parasites were disrupted by using a Dounce homogenizer, and the nuclei that were recovered by centrifugation were lysed in 0.5 M KCl. After dialysis to reduce the salt concentration, the extract was fractionated by Econo Pac Q, heparin agarose, and mono Q column chromatography (full details will be published elsewhere). The recovered fractions were tested for kinetoplast DNA decatenation to locate DNA topoisomerase II, and the active fractions were combined, adjusted to 20% glycerol, and stored at −20°C.

Mammalian cytotoxicity assays. Human Jurkat leukemia cells were routinely grown in RPMI 1640 medium containing 10% fetal calf serum. Cell numbers doubled every 24 h. Drugs from stock solutions in dimethyl sulfoxide were added to separate cultures at different concentrations, and the effects on cell growth (IC_{50}) and viability were measured over 72 h by using an improved Neubauer hemacytometer. The dimethyl sulfoxide concentrations were always less than 0.1%, which did not affect growth. Growth assays were performed in duplicate. IC_{50} data are presented in Table 1.

RESULTS

Previous studies (6) showed that 9-anilinoacridines bearing 1′-NH-alkyl substituents possess various levels of activity (0.4 to 2.6 μM) against chloroquine- and pyrimethamine-resistant *P. falciparum*. We have extended the range of 1′ substituents to include OH and CH_2 (alkylamino) compounds (compounds 1 to 11 in Table 1). In addition, since earlier studies (6) showed that 3-amino substitution in the acridine ring leads to increased potency, a number of 3-NH_2 and 3,6-diNH_2 analogs of compounds with acceptable 1′ substituents were also evaluated (compounds 12 to 19 in Table 1). Table 1 summarizes the structures, relevant physicochemical properties, and activities of the various drugs tested in vitro against *P. falciparum* and the Jurkat mammalian cell line.

Since 9-anilinoacridines are potent inhibitors of DNA topoisomerase II in mammalian cells, one of the more potent analogs (compound 13; 3,6-diamino-1′-amino-9-anilinoacridine) and pyronaridine (compound 22) were also tested for their inhibitory activities against DNA topoisomerase II in extracts of *P. falciparum*. Figure 1 shows the effect of compound 13 on the decatenation of kinetoplast DNA by partially purified *P. falciparum* DNA topoisomerase II. A concentration of 5 μM was slightly inhibitory to the enzyme (Fig. 1, lane 4), while complete inhibition of decatenation was obtained at or above a concentration of 10 μM (Fig. 1, lane 5). Pyronaridine (compound 22) also inhibited *P. falciparum* DNA topoisomerase II, with complete inhibition requiring 11 μM pyronaridine (data not shown).

DISCUSSION

The data in Table 1 show that 1′ substituents on 9-anilinoacridines have a moderate influence on in vitro antimalarial potency. One aim of the present study was to determine the influence of 1′-NH-R substituents, which are known to permit ready metabolic oxidation of 9-anilinoacridines to the corresponding diimines (10). These then undergo rapid hydrolysis to the quinoneimines and 1,4-addition by nucleophiles to form 5′ and 6′ conjugates (10, 12). However, the data show little difference in potency between compounds capable of metabolism by this route (compounds 1 to 4 and 7) and those which are not (compounds 5, 6, and 8 to 11), suggesting that it is not a determinant of activity. In fact, the 1′-CH_2-R analogs were consistently the most potent of the mono-substituted compounds.

Because of the increase in antimalarial potency by 3-NH_2 acridine substitution shown earlier (6), a series of 3-NH_2 and 3,6-diNH_2 analogs of selected 1′-substituted compounds were also evaluated. The 3-NH_2 compounds (compounds 12, 14, 16, and 18) were, on average, no more potent than the corresponding compounds (compounds 1, 2, 5, and 10) with an unsubstituted acridine, although there were individual differences (e.g., compounds 1 and 12). However, the 3,6-diNH_2 compounds (compounds 13, 15, 17, and 19) were all significantly (ca. 10-fold) more potent against *P. falciparum*, with IC_{50}s in the low nanomolar range, approaching that of the new antimalarial agent pyronaridine (compound 22) (IC_{50}, 2.7 nM).

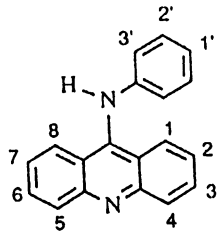
Some broad relationships between drug properties and in vitro antimalarial activity can be discerned. Compounds 1 to 11, with different C-1′ substituents, have acridine pKa values ranging from 7.7 to 8.5, which are sufficiently high and similar to ensure reasonable ionization at physiological pH, and the DNA binding constants are sufficiently similar to suggest that these parameters do not strongly influence activity. In contrast, the two most lipophilic compounds (compounds 3 and 4) were least active against *P. falciparum*, suggesting that high lipophilicity is an undesirable feature and that high hydrophilicity is necessary to obtain highly active drugs. The data for the next two compounds (compounds 12 and 13) agree with these conclusions. The pKa values of these two compounds correlated with their activities and they are hydrophilic because of their complete ionization at physiological pH, with the most hydrophilic compound being the 3,6-diamino-1′-amino derivative (compound 13) with an IC_{50} of 25 nM. Overall, the most active compounds (IC_{50}s, <0.3 μM) are all relatively hydrophilic molecules with negative R_m values, and this appears to be the most important global parameter for good antimalarial activity. Although the antimalarial agent pyronaridine possesses a relatively weakly basic acridine-like chromophore, it is also very hydrophilic because of its 1-aza atom and doubly-basic side chain.

Structure-activity relationships for the mammalian cytotoxicities of the compounds (measured as IC_{50}s against cultured Jurkat human leukemia cells) were quite different, with the 3,6-diNH_2 compounds being considerably less cytotoxic than either the 3-NH_2-substituted or unsubstituted analogs. Inhibition of Jurkat cell growth was usually accompanied by considerable enlargement of the cells, possibly indicating inhibition in the G_2 phase of the cell cycle, as occurs with other known DNA topoisomerase inhibitors (15).

The opposing effects of 3,6-diNH_2 substitution (enhanced toxicity against *P. falciparum* but reduced mammalian cell cytotoxicity) are reflected in the large increase in the in vitro therapeutic index (IVTI; defined as IC_{50} for Jurkat cells/IC_{50} for *P. falciparum*) seen for the 3,6-diNH_2 compounds. The IVTI of compound 13 (IVTI, 600), is superior to that of pyronaridine (compound 22) (IVTI, 550), and indicates that further exploration of this and related substitution patterns in 9-anilinoacridines is warranted.

The overall structure-activity relationships of the 9-anilinoacridines for inhibition of malarial parasites are likely to reflect effects on drug penetration as well as DNA topo-

TABLE 1. Physicochemical and biological data for antimalarial 9-anilinoacridines



Compound no.	Anilino substituent	Acridine substituent	R_m[a]	pKa of acridine[b]	DNA binding[c]	Jurkat leukemia $IC_{50}J$ (μM)[d]	*P. falciparum* $IC_{50}P$ (μM)[e]	IVTI ($IC_{50}J/IC_{50}P$)[f]
1	1′-NH_2	H	−0.08	8.36	2.0	0.6	1.5	0.4
2	1′-$NHCH_3$	H	0.24	8.42	3.5	1.5	0.4	3.75
3	1′-$NH(CH_2)_2CH_3$	H	0.77	8.24	2.2	4.0	2.4	1.65
4	1′-$NH(CH_2)_5CH_3$	H	1.03	[8.2][g]	2.7	16.0	2.6	6.15
5	1′-$N(CH_3)_2$	H	0.66	8.46	3.2	1.6	0.47	3.4
6	1′-$N(CH_3)CH_2CH_3$	H	0.66	[8.5][g]	0.91	4.0	0.6	6.65
7	1′-OH	H	0.32	[8.2][g]	1.9	2.5	2.1	1.2
8	1′-CH_2NH_2	H	−0.91	[7.7][g,h]	0.37	3.3	0.23	14.35
9	1′-CH_2NHCH_3	H	−0.9	[7.7][g,i]	0.3	1.5	0.24	6.25
10	1′-$CH_2N(CH_3)_2$	H	−0.87	[7.7][g,i]	0.25	7.5	0.15	50
11	1′-$CH_2CH_2NH_2$	H	−0.69	[7.7][g,j]	0.24	1.4	0.32	4.4
12	1′-NH_2	3-NH_2	−0.48	10.73	0.62	1.0	0.1	10
13	1′-NH_2	3,6-diNH_2	−1.21	10.99	0.74	15.0	0.025	600
14	1′-$NHCH_3$	3-NH_2	−0.07	9.95	0.3	0.5	0.32	1.55
15	1′-$NHCH_3$	3,6-diNH_2	−0.65	[11.0][g]	0.8	11.5	0.16	72
16	1′-$N(CH_3)_2$	3-NH_2	0.31	[9.95][g]	0.6	<1	0.29	<3.45
17	1′-$N(CH_3)_2$	3,6-diNH_2	0.3[k]	[11.0][g]	0.55	5.0	0.034	147
18	1′-$CH_2N(CH_3)_2$	3-NH_2	−0.1[k]	[9.15][g,i]	0.65	2.5	0.25	10
19	1′-$CH_2N(CH_3)_2$	3,6-diNH_2	−1.4[k]	[10.3][g,i]	0.6	>20	0.04	>500
20	1′-$NHSO_2CH_3$, 3′-OCH_3	H	0.18	7.43	10.8	<1.0	0.5	<2
21	1′-$NHSO_2CH_3$, 2′-OCH_3	H	0.3	7.03	3.6	6.0	26.0	0.2
22	Pyronaridine		>−1.5[k]	[5.9][g,l]	0.17	1.5	0.0027	555

[a] Lipophilicity as R_m on liquid-liquid thin-layer chromatography for cations.
[b] pKa in 20% dimethylformamide–water; add 0.59 Units for aqueous value.
[c] Concentration of drug (μM) to displace 50% of the bound ethidium from calf thymus DNA. Inversely related to DNA binding constant.
[d] $IC_{50}J$, concentration of drug to reduce growth of leukemia cells to 50% of controls by using a 72-h continuous exposure.
[e] $IC_{50}P$, concentration of drug to reduce the number of parasites (*P. falciparum* K1) to 50% of the number of controls (2).
[f] IVTI, in vitro therapeutic index = $IC_{50}J/IC_{50}P$.
[g] Values in brackets are estimated acridine pKa (5).
[h] Side chain pKa estimated as 9.3.
[i] Side chain pKa estimated as 8.9.
[j] Side chain pKa estimated as 9.35.
[k] Estimated R_m.
[l] Side chain pKa estimated as 8.9.

isomerase II inhibition. While both compound 13 and pyronaridine (compound 22) inhibit *P. falciparum* DNA topoisomerase II in vitro, structure-activity relationships for activity against topoisomerase II have not yet been determined for all of the compounds. However, with the availability of an in vitro assay for the topoisomerase II from *P. falciparum*, it should now be possible to identify any structural features of the drugs that enhance inhibition of the enzyme. Previous studies (7) have shown that it is possible to develop 9-anilinoacridine variants with selective activity against isozymes of mammalian DNA topoisomerase II, and it is expected that the *P. falciparum* topoisomerase II should be at least as distinct as these isozymes. Interestingly, the concentrations of both compound 13 and pyronaridine required to inhibit the decatenation of *P. falciparum* DNA topoisomerase II were higher than those observed to inhibit whole-cell growth. However, other studies (16) have shown that the 9-anilinoacridine 4′-[9-(acridinyl)amino]methansulfon-*m*-anisidide (*m*-AMSA) is rapidly taken up by L1210 cells, and at concentrations exceeding 10 μM, the uptake is proportionately greater than the increase in external drug concentration. Zwelling et al. (16) suggested that a cooperative uptake process occurs at high *m*-AMSA concentrations.

The data from the present study show that the overall hydrophilicity and the high basicity of 9-anilinoacridines are more important determinants of parasite inhibition than acridine ring pKa or DNA binding. This dependence on hydrophilic character seems likely to reflect drug penetration factors rather than effects on topoisomerase II inhibition. Malaria parasites alter the membranes of host erythrocytes to obtain nutrients which might favor the accumulation of more hydrophilic or highly charged compounds and provide another opportunity for distinguishing between host and

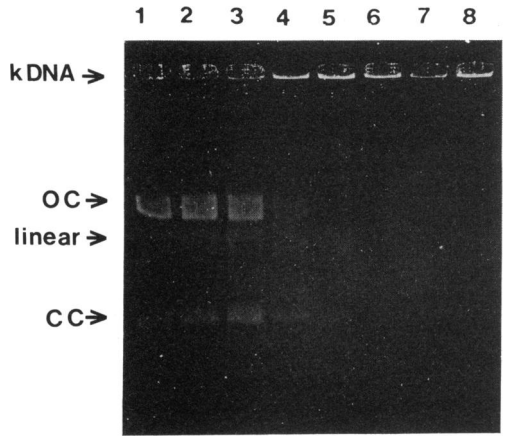


FIG. 1. Effect of 3,6-diamino-1′-amino-9-anilinoacridine (compound 13) on the decatenation activity of partially purified DNA topoisomerase II from *P. falciparum*. The reaction contained 1 U of enzyme activity (that amount of enzyme which decatenated 50% of kinetoplast DNA [0.1 μg] in 30 min at 37°C). Lanes 1 to 8, the presence of 0, 5×10^{-7}, 1×10^{-6}, 5×10^{-6}, 1×10^{-5}, 5×10^{-5}, 1×10^{-4}, and 5×10^{-4} M compound 13, respectively. kDNA, kinetoplast DNA; OC, nicked open circular kinetoplast DNA; linear, linearized kinetoplast DNA; CC, relaxed closed circular kinetoplast DNA.

parasite (1, 9). These observations are a useful starting point from which to develop even more effective drugs of this series.

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