2007

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Original Publication Citation

Kelly, J. X., Smilkstein, M. J., Cooper, R. A., Lane, K. D., Johnson, R. A., Janowsky, A., . . . Riscoe, M. (2007). Design, synthesis, and evaluation of 10-N-substituted Acridones as novel chemosensitizers in *Plasmodium falciparum*. *Antimicrobial Agents and Chemotherapy, 51*(11), 4133-4140. doi:10.1128/aac.00669-07

ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 2007, p. 4133–4140 Vol. 51, No. 11
0066-4804/07/$08.00+0 doi:10.1128/AAC.00669-07

Design, Synthesis, and Evaluation of 10-N-Substituted Acridones as Novel Chemosensitizers in *Plasmodium falciparum*▽

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Received 22 May 2007/Returned for modification 25 July 2007/Accepted 27 August 2007

A series of novel 10-N-substituted acridones, bearing alkyl side chains with tertiary amine groups at the terminal position, were designed, synthesized, and evaluated for the ability to enhance the potency of quinoline drugs against multidrug-resistant (MDR) *Plasmodium falciparum* malaria parasites. A number of acridone derivatives, with side chains bridged three or more carbon atoms apart between the ring nitrogen and terminal nitrogen, demonstrated chloroquine (CQ)-chemosensitizing activity against the MDR strain of *P. falciparum* (Dd2). Isobologram analysis revealed that selected candidates demonstrated significant synergy with CQ in the CQ-resistant (CQR) parasite Dd2 but only additive (or indifferent) interaction in the CQ-sensitive (CQS) D6. These acridone derivatives also enhanced the sensitivity of other quinoline antimalarials, such as desethylchloroquine (DCQ) and quinine (QN), in Dd2. The patterns of chemosensitizing effects of selected acridones on CQ and QN were similar to those of verapamil against various parasite lines with mutations encoding amino acid 76 of the *P. falciparum* CQ resistance transporter (PfCRT). Unlike other known chemosensitizers with recognized psychotropic effects (e.g., desipramine, imipramine, and chlorpheniramine), these novel acridone derivatives exhibited no demonstrable effect on the uptake or binding of important biogenic amine neurotransmitters. The combined results indicate that 10-N-substituted acridones present novel pharmacophores for the development of chemosensitizers against *P. falciparum*.

Malaria remains a major world health problem and continues to be a leading cause of morbidity and mortality, particularly in developing countries (64). The devastating situation is largely attributed to and aggravated by the emergence and spread of multidrug-resistant (MDR) *Plasmodium falciparum*, the cause of the most deadly form of malaria (3, 28, 47, 67). Chloroquine (CQ) (Fig. 1) and other quinoline antimalarials, including the naturally occurring quinine (QN) (Fig. 1), were among the most successful chemotherapeutic agents for treatment of malaria for decades, if not centuries. Unfortunately, at present, resistance to CQ exists virtually wherever *P. falciparum* does, making it essentially useless in nearly all regions where malaria is endemic (3, 22, 63, 67). Although resistance to pregnancy-safe QN is far less extensive, reports of QN resistance are steadily increasing (3, 32, 48, 49, 53, 67).

In the 1980s, artemisinin-based drugs were not readily available. The lack of new effective drugs heightened the urgency for quick fixes to restore the usefulness of CQ and to counter the spread of CQ resistance. The discovery of MDR chemosensitizers (or so-called "resistance reversal agents") in the cancer research field stimulated a study by Martin et al., which revealed that the calcium channel blocker verapamil (Fig. 1) also restored CQ sensitivity to MDR *P. falciparum* parasites (39). Since then, many structurally and functionally diverse compounds have been identified and reported to demonstrate chemosensitization activity against malaria parasites, with antihistamines (e.g., chlorpheniramine) (Fig. 1) (4, 6, 42, 43, 46, 55) and tricyclic antidepressants (e.g., desipramine) (Fig. 1) (5, 8, 10, 11, 13, 40, 51, 57) among the most effective and best studied (27, 59).

While the mechanism of chemosensitization is not fully understood, recent studies suggest that mutations in the *P. falciparum* CQ resistance transporter (PfCRT) protein, particularly amino acid substitutions at position 76, may play key roles in the mode of action of verapamil (14, 18, 37). Structure-activity profiling and three-dimensional quantitative structure-activity relationship (QSAR) studies by Bhattacharjee and colleagues revealed a pharmacophore with critical features for potent CQ-chemosensitizing activity, which consists of two aromatic hydrophobic groups and a hydrogen bond acceptor site at the side chain, preferably on a nitrogen atom (8, 9, 25).

Our previous work described functionalized tricyclic xanthones that exert their antimalarial activities by accumulation in the acidic digestive vacuole of the parasite and formation of soluble complexes with heme (29, 30, 33–35, 50, 65). Here, we switched to the acridone nucleus to facilitate the attachment of a suitable R group (e.g., alkyl amine) at the 10-N position for chemosensitization function (Fig. 2). A further motivation for switching to the acridone system is for the design of a dually functional agent, with structural modifications to facilitate binding to heme and to the central nitrogen atom to introduce chemosensitization. The present paper focuses attention on functionalizing the acridone nucleus for the chemosensitization phenomenon. A series of novel 10-N-substituted acri-

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▽ Published ahead of print on 10 September 2007.

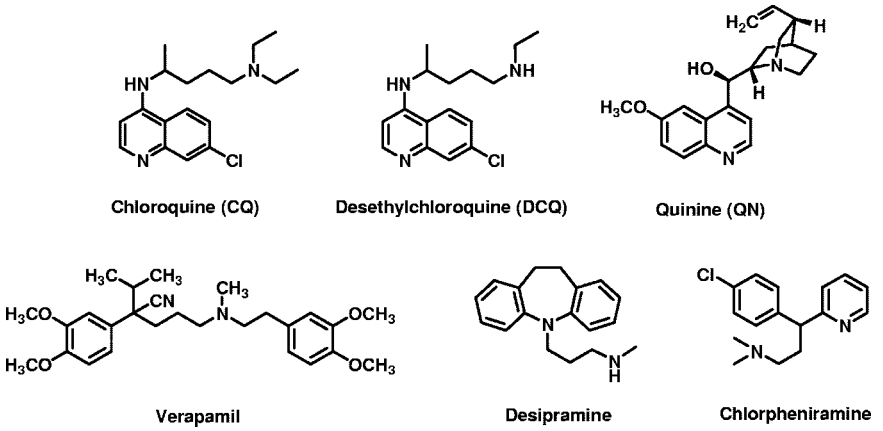

FIG. 1. Chemical structures of selected antimalarials and chemosensitizers.

dones (Fig. 3), bearing alkyl side chains (with lengths ranging from two to eight carbons) with tertiary amino groups at the terminal position, were designed, synthesized, and evaluated for the ability to enhance the potencies of quinoline drugs against MDR malaria parasites.

MATERIALS AND METHODS

Chemicals. CQ, QN, verapamil, desipramine, chlorpheniramine, and 9(10H)-acridone were purchased from Sigma-Aldrich Company (St. Louis, MO). Desethylchloroquine (DCQ) was a generous gift from Dennis Kyle of the Walter Reed Army Institute of Research (Silver Spring, MD).

Synthesis of 10-N-substituted acridones. Methods for the chemical syntheses of 10-N-substituted acridones are illustrated in Fig. 4. The two-carbon chain derivative was prepared from 9(10H)-acridone by refluxing with 2-(diethylamino)ethyl chloride hydrochloride in anhydrous acetone in the presence of K_2CO_3. N-alkylation of the ring nitrogen with longer side chains ($n > 2$) was achieved in two steps. First was the reaction of 9(10H)-acridone with 1,*n*-chlorobromoalkanes in the presence of potassium carbonate in anhydrous acetone under reflux conditions to yield the corresponding *N*-10-(*n*-chloroalkyl)-acridones. Following isolation of this intermediate, the *N*-10-(*n*-chloroalkyl)-acridones were stirred with diethylamine in dimethyl sulfoxide in the presence of NaI, leading to formation of the desired alkylamino-substituted acridones. The final product was purified by crystallization or column chromatography and analyzed for identity and purity by proton nuclear magnetic resonance (NMR) and combustion analysis prior to use.

Cultivation of *P. falciparum*. Two strains of *P. falciparum* were used in the initial study. The CQ-sensitive (CQS) clone D6 and the CQ-resistant (CQR) and MDR clone Dd2 were obtained from the Malaria Research and Reference Reagent Resource Center (Manassas, VA). The parasites were cultured according to the method of Trager and Jensen (58) with minor modifications. The cultures were maintained in human erythrocytes (Lampire Biological Laboratories, Pipersville, PA); suspended at 2% hematocrit in RPMI 1640 (Sigma) containing 0.5% Albumax (Invitrogen Corporation, Carlsbad, CA), 45 μg/liter hypoxanthine (Lancaster), and 50 μg/liter gentamicin (Invitrogen); and incubated at 37°C under a gas mixture of 5% O_2, 5% CO_2, and 90% N_2.

In vitro drug susceptibility testing in *P. falciparum*. In vitro antimalarial activity was determined by a malaria SYBR Green I-based fluorescence (MSF) method described previously by Smilkstein et al. (54) with slight modification (66). Stock solutions of each test drug were prepared in sterile distilled water at a concentration of 10 mM. The drug solutions were serially diluted with culture medium and distributed to asynchronous parasite cultures on 96-well plates in quadruplicate in a total volume of 100 μl to achieve 0.2% parasitemia with a 2% hematocrit in a total volume of 100 μl. Automated pipetting and dilution were carried out with a programmable Precision 2000 robotic station (Bio-Tek, Winooski, VT). The plates were then incubated for 72 h at 37°C. After incubation, 100 μl of lysis buffer with 0.2 μl/ml SYBR Green I (54, 66) was added to each well. The plates were incubated at room temperature for an hour in the dark and then placed in a 96-well fluorescence plate reader (Spectramax Gemini-EM; Molecular Diagnostics) with excitation and emission wavelengths at 497 nm and 520 nm, respectively, for measurement of fluorescence. The 50% inhibitory concentration (IC_{50}) was determined by nonlinear regression analysis of logistic dose-response curves (GraphPad Prism software).

Alamar Blue assay for mammalian cell viability. The general cytotoxic effects of acridone derivatives on host cells were assessed by functional assay as described previously (1, 66, 69), using murine splenic lymphocytes induced to proliferate and differentiate by concanavalin A. Splenic lymphocytes isolated from C57BL/6J mice were washed twice in RPMI 1640 medium and resuspended in complete RPMI containing 10% fetal bovine serum, 50 μg/ml penicillin/streptomycin, 50 μM β-mercaptoethanol, and 1 μg/ml concanavalin A. Cells (100 μl/well) then were seeded into 96-well flat-bottom tissue culture plates containing drug solutions (100 μl) serially diluted with complete culture medium to a final cell density of 2×10^5 per well. The plates were then incubated for 72 h in a humidified atmosphere at 37°C and 5% CO_2. An aliquot of a stock solution of resazurin (Alamar Blue, prepared in 1× phosphate-buffered saline) was then added at 20 μl per well (final concentration, 10 μM), and the plates were returned to the incubator for another 24 h. After this period, the fluorescence in each well was measured in a Gemini EM plate reader with an excitation wavelength at 560 nm and an emission wavelength at 590 nm. IC_{50}s were determined by nonlinear regression analysis of logistic concentration-fluorescence intensity curves (GraphPad Prism software).

Evaluation of the in vitro chemosensitizing activity of acridones. For drug interaction studies, a fixed subinhibitory concentration (500 nM) of the selected

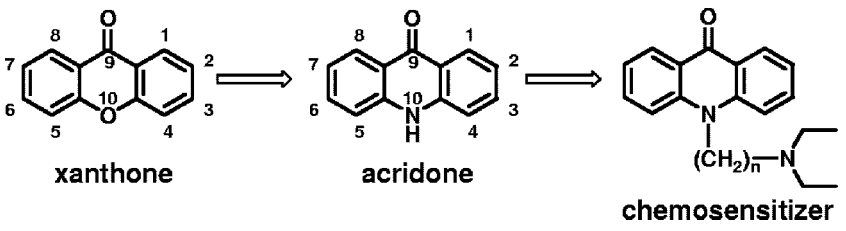

FIG. 2. Design pathway of acridone chemosensitizers.

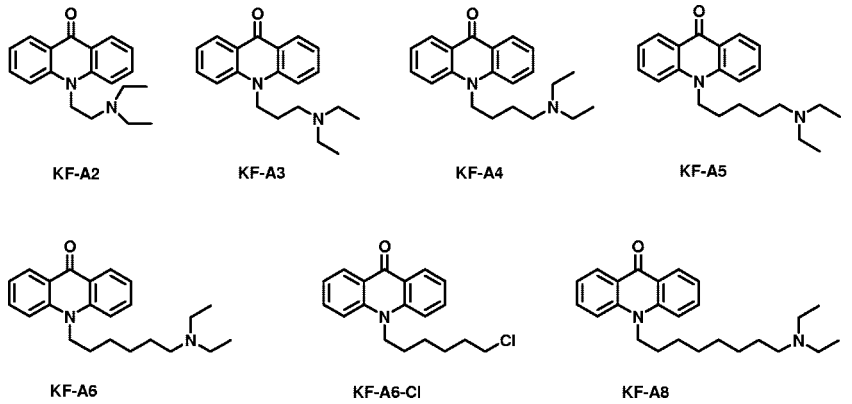

FIG. 3. Chemical structures of profiled 10-N-substituted acridone derivatives.

acridone was combined with the pre-serially diluted quinoline-containing drug. The effects of the acridones on the activities of quinoline antimalarials were assessed by comparing concentration-response curves for quinoline alone and in the presence of the selected acridone. The data were transformed and expressed as the response modification index (RMI). The RMI is defined as the ratio of the IC_{50} of drug A in the presence of drug B to the IC_{50} of drug A alone, where drug A is the quinoline antimalarial and drug B is the selected acridone. An RMI of 1.0 indicates no change in the IC_{50} of drug A upon addition of drug B. An RMI of less than 1.0 represents chemosensitization (including possible synergy), and an RMI of more than 1.0 represents antagonism (42). This method is widely used in the field as an initial screening tool for chemosensitization effect, mainly due to its ease of operation.

Effects of PfCRT mutations on chemosensitization activities of KF-A6 on CQ and QN. Details of the single-step selection of the *P. falciparum pfcrt* mutant lines $106/1^{76I}$, $106/1^{76N}$, and $106/1^{76T}$ have been described previously by Cooper et al. (14). The IC_{50}s of drugs alone or in combination (e.g., CQ/KF-A6, CQ/verapamil, QN/KF-A6, and QN/verapamil) were determined by a modified MSF method in which 1% parasitemia and 1% hematocrit were used.

Fixed-ratio isobologram analysis of drug interactions between CQ and acridones. For definitive determination of synergy, selected acridones were tested in combination with CQ using a modified fixed-ratio method described by Fivelman et al. (20). After determination of the IC_{50}s for selected drugs, stock solutions were prepared with each drug at concentrations such that the final concentration in our 96-well drug susceptibility assay after four or five twofold dilutions approximated the IC_{50}. If we call these stock solutions drug A and drug B, then six final stock solutions were prepared from this initial stock: drug A alone, drug B alone, and volume-volume mixtures of drugs A and B in the following ratios: 4:1, 3:2, 2:3, and 1:4. Twofold dilutions of each of the six final stock solutions were performed robotically across a 96-well plate in quadruplicate. Subsequent steps were typical of the standard drug susceptibility methods as described above. Initial data analysis yielded the intrinsic dose-response curve for each drug alone and four different fixed-ratio combination dose-response curves, with corresponding IC_{50}s. The fractional inhibitory concentrations (FICs) were then calculated by the following formulas: FIC (A) = IC_{50} of drug A in combination/IC_{50} of drug A alone; FIC (B) = IC_{50} of drug B in combination/IC_{50} of drug B alone; FIC index = FIC (A) + FIC (B). The isobolograms were constructed by plotting a pair of FICs for each combination of CQ and the selected compound. Traditionally, an interpretation of a straight diagonal line (FIC index = 1) on the isobologram indicates a purely additive effect between the two drugs. A concave curve below the line (FIC index < 1.0) indicates synergy of the combination, while a convex curve above the line (FIC index > 1.0) indicates antagonism. However, conventions have been adopted that define a FIC index of ≤0.5 as synergy, a FIC index of ≥4.0 as antagonism, and a FIC index between 0.5 and 4.0 as no interaction or indifference (2, 41). Isobologram analysis with fixed-ratio combinations is a more sophisticated and disciplined method to establish the definitive determination and the degree of synergy (or antagonism) for drug interaction.

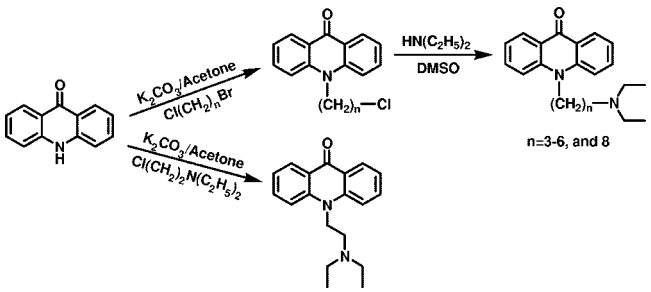

FIG. 4. Synthetic routes for 10-N-substituted acridone derivatives.

Effects of acridone derivatives on inhibition of substrate uptake by recombinant hDAT, hSERT, and hNET transporters. The uptake assays employed for this study were adapted from the method of Eshleman et al. (17). Human embryonic kidney (HEK)-human dopamine (hDAT), -serotonin (hSERT), and -norepinephrine (hNET) cells were grown on 150-mm-diameter tissue culture dishes. The medium was removed, and the plates were washed twice with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline. The cells were gently scraped from the plates in Krebs-HEPES assay buffer, and cell clusters were separated by trituration with a pipette. Aliquots (50 μl) of the suspended cells were added to assay tubes containing drugs and Krebs-HEPES assay buffer in a final volume of 0.5 ml. Competition experiments were conducted with triplicate determinations for each point. After a 10-min preincubation in a 25°C water bath, ^{3}H-labeled neurotransmitter (20 nM [final concentration] of [^{3}H]DA (dopamine), [^{3}H]5-HT (serotonin), or [^{3}H]NE (norepinephrine), 56, 26.9, or 60 Ci/mmol, respectively) was added, and the assay mixture was incubated for 10 min at 25°C. The reaction was terminated by filtration through Wallac filtermat A filters presoaked in 0.05% polyethylenimine, using a Tomtec cell harvester. Scintillation fluid was added to each filtered spot, and radioactivity remaining on the filters was determined using a Wallac 1205 Betaplate scintillation counter. Specific uptake was defined as the difference in uptake observed in the absence and presence of 5 μM mazindol (hDAT and hNET) or 5 μM imipramine (hSERT). Prism software was used to analyze the uptake data.

Effects of acridone derivatives on radioligand binding to histamine receptors. [^{3}H]pyrilamine binding assays were conducted using the modified radioligand binding methods of Janowsky et al. (31). [^{3}H]pyrilamine and recombinant human histamine receptor subtype 1, expressed in CHO cells, were purchased from Perkin-Elmer Life and Analytical Sciences (Boston, MA). [^{3}H]pyrilamine binding assay mixtures contained an aliquot of a membrane preparation (approximately 50 μg protein), drug, and [^{3}H]pyrilamine (5 nM final concentration) in a final volume of 500 μl. Tris-HCl buffer (50 mM; pH 7.4) was used for all assays. Specific binding was defined as the difference in binding observed in the presence and absence of 10 μM hydroxyzine. Membranes were preincubated with drugs at room temperature for 10 min before the addition of [^{3}H]pyrilamine unless otherwise indicated. The reaction mixture was incubated for 90 min at room temperature in the dark and was terminated by filtration through Wallac Filtermat A filters (the filters were soaked in 0.05% polyethyeneimine for 15 min prior to filtration), using a 96-well Tomtec cell harvester. Scintillation fluid (50 μl) was added to each filtered spot, and radioactivity remaining on the filter was determined using a Wallac 1205 Betaplate or 1405 microBeta scintillation counter. Competition experiments were conducted with duplicate determinations for each

TABLE 1. Intrinsic in vitro antimalarial activities, in vitro cytotoxicities, and biophysical properties of selected chemosensitizers

Compound	IC_{50}[a] (μM) for *P. falciparum*		IC_{50}[b] (μM) for MSLCs	cLogP[c]	Calculated pK_a[d]
	D6	Dd2			
KF-A2	11.8 ± 0.8	7.5 ± 1.0	28.5 ± 1.8	3.11	9.81
KF-A3	8.1 ± 0.5	1.6 ± 0.3	22.5 ± 1.0	3.36	10.28
KF-A4	7.6 ± 0.7	10.2 ± 1.5	34.5 ± 2.8	3.37	10.46
KF-A5	3.7 ± 0.3	5.8 ± 2.2	30.5 ± 3.0	3.90	10.53
KF-A6	3.2 ± 0.4	2.7 ± 0.7	26.4 ± 2.3	4.43	10.56
KF-A8	2.6 ± 0.2	1.8 ± 0.2	17.6 ± 2.1	5.49	10.65
KF-A6-Cl	1.3 ± 0.1	2.6 ± 0.3	59.5 ± 4.8	4.29	
Verapamil	1.3 ± 0.3	2.0 ± 0.2	55.0 ± 6.2	4.47	8.6
Desipramine	11.4 ± 0.5	16.6 ± 1.5	22.9 ± 1.6	4.47	10.4
Chlorpheniramine	61.2 ± 3.2	3.9 ± 0.4	33.4 ± 2.5	3.15	10.2

[a] Values are the means ± standard errors of the mean for three independent experiments, each in quadruplicate.
[b] Values are the means ± standard errors of the mean for two independent experiments, each in quadruplicate. MSLCs, murine splenic lymphocytes.
[c] cLogP (calculated *n* = octanol/water partition coefficient) values were calculated with ChemDraw Ultra 8.0 software.
[d] pK_a values were calculated with ChemSketch I-lab.

point. GraphPad Prism software was used to analyze all kinetic and competition binding data.

RESULTS

Intrinsic antimalarial activity, effect on mammalian cell viability, and biophysical properties. Each of the acridone derivatives and the prototypical chemosensitizers was tested against a CQS strain (D6), as well as an MDR strain (Dd2), of *P. falciparum*. As shown in Table 1, the acridone derivatives exhibited moderate intrinsic antimalarial activity, with IC_{50}s in the low micromolar range. The intrinsic activities of acridone derivatives were comparable to those of verapamil, desipramine, and chlorpheniramine against the selected strains of *P. falciparum*.

These 10-N-substituted acridones did not exhibit profound in vitro cytotoxicity against the proliferation of mitogen-induced splenic lymphocytes, with IC_{50}s ranging from 17.6 to 59.5 μM (Table 1).

The calculated biophysical properties of these acridones suggest that the compounds are moderately lipophilic at physiological pH, with cLogP values from 3.11 to 5.49 (Table 1). As indicated by pK_a values ranging from 9.81 to 10.65 (Table 1), the acridone derivatives with the ionizable alkyl amine side chains, KF-A2, KF-A3, KF-A4, KF-A5, KF-A6, and KF-A8, exist as a mixture of unprotonated and protonated forms at pH 7 (physiological conditions) and predominantly as positively charged forms at pH values close to 5 (approximating conditions of the parasite food vacuole) (7, 16, 23, 24, 26, 36, 68).

Chemosensitization of CQ in MDR *P. falciparum*. To examine the abilities of the acridone derivatives to enhance CQ potency against *P. falciparum*, the IC_{50}s of CQ were determined in combination with 500 nM of the test acridone against the CQR strain Dd2 and the CQS strain D6 (Table 2). At the selected drug concentration, the acridones with a tertiary amine at the ends of the various-length carbon chains exhibited CQ-chemosensitizing activities against an MDR strain of *P. falciparum* (Dd2) ranging from indifferent (KF-A2) to partial (KF-A3 and KF-A8) to considerable (KF-A4, KF-A5, and KF-A6). In the presence of 500 nM KF-A6, the antimalarial activity of CQ was potentiated >80-fold in the MDR strain (Dd2) to a level (19.0 nM) similar to that observed for the CQS strain, D6 (14.7 nM). As revealed in Table 2, KF-A5 and KF-A6 exhibited a CQ-chemosensitization effect (against the CQR strain Dd2) superior to those of the well-studied chemosensitizers verapamil and chlorpheniramine and comparable to the effect of desipramine at the equivalent drug concentration. There was no apparent enhancement of the sensitivity of CQ against the CQS strain (D6) with either the acridone derivatives or the reference chemosensitizers (Table 2).

Interestingly, 10-(6-chlorohexyl)-acridone (KF-A6-Cl), bearing the N-10 alkyl chain but terminated with a chlorine atom (i.e., instead of the terminal amine group), was without CQ chemosensitization effectiveness against the CQR strain Dd2, thereby demonstrating the critical role of the terminal amine for the CQ chemosensitization effect (Table 2).

Isobolar analysis of drug interactions between CQ and selected chemosensitizers. To assess whether the degree of chemosensitization met the current definition of synergy, in vitro interaction studies between CQ and selected acridone derivatives against both CQR (Dd2) and CQS (D6) parasites were evaluated by the more sophisticated isobolar analysis using the fixed-ratio combination method (Fig. 5). In these studies, significant synergy was observed in the combinations of CQ/KF-A4, CQ/KF-A5, and CQ/KF-A6 against the CQR parasite

TABLE 2. In vitro chemosensitizing effects of acridone derivatives and other known chemosensitizers on CQ against *P. falciparum*

Drug combination	Dd2		D6	
	IC_{50}[a] (nM)	RMI[b]	IC_{50}[a] (nM)	RMI[b]
CQ alone	106.4 ± 7.5		14.7 ± 1.8	
CQ + 500 nM KF-A2	113.3 ± 9.6	1.06	13.7 ± 1.1	0.93
CQ + 500 nM KF-A3	46.7 ± 4.2	0.44	14.7 ± 1.6	1.00
CQ + 500 nM KF-A4	42.8 ± 2.2	0.40	15.4 ± 0.8	1.05
CQ + 500 nM KF-A5	30.8 ± 4.8	0.29	13.7 ± 0.9	0.93
CQ + 500 nM KF-A6	19.0 ± 1.7	0.18	13.1 ± 0.9	0.89
CQ + 500 nM KF-A8	48.9 ± 3.6	0.46	14.3 ± 1.2	0.97
CQ + 500 nM KF-A6-Cl	143.8 ± 14.4	1.35	16.0 ± 1.4	1.09
CQ + 500 nM verapamil	56.8 ± 6.0	0.53	14.1 ± 1.2	0.96
CQ + 500 nM desipramine	18.9 ± 2.1	0.18	16.0 ± 1.1	1.09
CQ + 500 nM chlorpheniramine	44.3 ± 2.6	0.42	15.0 ± 1.8	1.02

[a] Values are the means ± standard errors of the mean for three independent experiments, each in quadruplicate.
[b] RMI is calculated as the ratio of the IC_{50} of CQ in the presence of chemosensitizer to the IC_{50} of CQ alone.

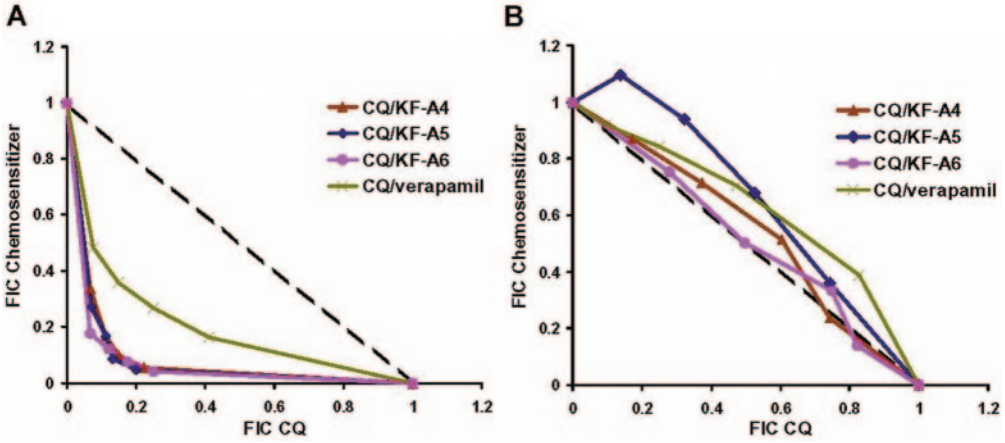

FIG. 5. Isobologram of the in vitro interaction between CQ and chemosensitizers against the MDR strain of *P. falciparum*, Dd2 (A), and the CQS strain of *P. falciparum*, D6 (B). The *x* axis represents the FICs of CQ, and the *y* axis represents the FICs of selected chemosensitizers. Each point represents a FIC of the respective chemosensitizer and its corresponding FIC of CQ. The diagonal (dashed) line indicates the hypothetical additive drug effect.

Dd2, with mean FIC indices of 0.3, 0.27, and 0.25, respectively. The CQ/KF-A4, CQ/KF-A5, and CQ/KF-A6 combinations exhibited superior synergy compared to the reference drug combination CQ/verapamil (FIC index = 0.41) against the CQR parasite Dd2.

In contrast, as indicated by the additive (or indifferent) diagonal isobologram (Fig. 5), KF-A4, KF-A5, and KF-A6 did not exhibit synergism with CQ against the CQS parasite D6, with mean FIC indices of 1.1, 1.2, and 1.0, respectively. Similar results were observed for the CQ/verapamil combination (Fig. 5). These findings are consistent with the characteristics of classical chemosensitizers based on previous reports (39).

Chemosensitization to DCQ and QN in MDR *P. falciparum*. The lead acridone derivative KF-A6 enhances the sensitivity of other quinoline-containing antimalarials, DCQ (a major metabolite of CQ) and QN, in the MDR parasite Dd2 but has no effect on the CQS strain D6 (Table 3). In the presence of 500 nM KF-A6, the IC_{50} of DCQ against Dd2 was lowered more than 80% and the IC_{50} of QN against Dd2 was lowered to approximately the same level as that observed for the sensitive strain, D6.

Effects of PfCRT mutations on the chemosensitization activity of KF-A6 on CQ and QN. Recent studies have suggested that mutations in PfCRT, particularly amino acid substitutions at position 76, may play key roles in the mode of action of verapamil (14, 18, 37). Several novel CQR *pfcrt* mutant lines with different point mutations at codon 76 have been derived from a CQS parent line, $106/1^{K76}$ (14). This is a Sudanese isolate that contains six out of seven PfCRT mutations typically found in Old World CQR parasite lines but lacks the critical K76T polymorphism (14). For this study, mutant lines $106/1^{76I}$ and $106/1^{76N}$, along with $106/1^{76T}$, were chosen (due to their common isogenic background) to explore the effects of PfCRT mutations on the chemosensitization behavior of selected acridone derivatives.

Table 4 shows the effect of PfCRT position 76 mutations on the chemosensitizing activity of KF-A6 and verapamil when tested in combination with either CQ or QN. The results reveal mutation-specific changes in intrinsic sensitivity and chemosensitizing activity for all drugs. Verapamil and KF-A6 showed moderate intrinsic antimalarial activity, with micromolar IC_{50}s against the mutant lines $106/1^{76T}$ and $106/1^{76I}$. In the $106/1^{76N}$ line, however, a much greater intrinsic sensitivity to KF-A6 and verapamil was observed when the drugs were tested alone. The potency of CQ was enhanced by both drugs against all tested mutant lines, and the potency of QN was enhanced by both drugs against the mutant lines $106/1^{76T}$ and $106/1^{76N}$. KF-A6 was a more potent chemosensitizing agent (for both CQ and QN) than verapamil in these cases. Most significantly, the

TABLE 3. In vitro chemosensitizing effects of KF-A6 on DCQ and QN against *P. falciparum*

Drug combination	Dd2		D6	
	IC_{50}[a] (nM)	RMI[b]	IC_{50}[a] (nM)	RMI[b]
DCQ alone	372.0 ± 51.6		18.2 ± 1.5	
DCQ + 500 nM KF-A6	64.2 ± 3.7	0.17	18.5 ± 2.1	1.01
QN alone	59.8 ± 3.6		12.6 ± 1.0	
QN + 500 nM KF-A6	17.8 ± 1.1	0.30	12.7 ± 1.1	1.00

[a] Values are the means ± standard errors of the mean for three independent experiments, each in quadruplicate.
[b] RMI is calculated as the ratio of the IC_{50} of DCQ (or QN) in the presence of KF-A6 to the IC_{50} of DCQ (or QN) alone.

TABLE 4. Effects of PfCRT position 76 mutations on in vitro chemosensitization of CQ and QN by KF-A6 and verapamil in *P. falciparum*

Compound	IC_{50}[a] (nM) for *P. falciparum*		
	$106/1^{76T}$	$106/1^{76N}$	$106/1^{76I}$
Verapamil alone	4,053 ± 940.2	653.0 ± 92.6	7,074 ± 1407
KF-A6 alone	4,499 ± 609.7	596.0 ± 67.1	3,656 ± 410.9
CQ alone	102.2 ± 3.7	48.0 ± 5.9	127.0 ± 7.0
CQ + 500 nM verapamil	59.9 ± 3.5	31.5 ± 1.9	64.8 ± 2.6
CQ + 500 nM KF-A6	37.1 ± 2.5	29.6 ± 2.8	63.6 ± 2.4
QN alone	153.0 ± 16.1	60.6 ± 4.3	19.0 ± 0.8
QN + 500 nM verapamil	103.3 ± 5.7	39.2 ± 3.6	30.3 ± 1.5
QN + 500 nM KF-A6	91.8 ± 4.7	31.1 ± 1.9	35.5 ± 1.6

[a] Mean IC_{50}s from three to eight independent modified MSF assays (see Materials and Methods for details).

TABLE 5. Inhibition of [^{3}H]neurotransmitter uptake in HEK-hDAT, HEK-hSERT, and HEK-hNET cells and inhibition of [^{3}H]pyrilamine binding to histamine H1 receptor

Compound	IC_{50}[a] (μM)			
	[^{3}H]DA uptake in HEK-hDAT	[^{3}H]5-HT uptake in HEK-hSERT	[^{3}H]NE uptake in HEK-hNET	[^{3}H]pyrilamine binding H1
KF-A2	>10	>10	>10	
KF-A3	>10	>10	>10	
KF-A4	>10	>10	>10	
KF-A5	>10	7.8 ± 0.6	9.1 ± 1.1	
KF-A6	>10	3.5 ± 0.2	>10	3.6 ± 0.4
Desipramine	82.0 ± 6.3	0.064 ± 0.008	0.0042 ± 0.0004	
Imipramine	25.6 ± 1.5	0.008 ± 0.001	0.074 ± 0.009	
Cocaine[b]	0.13 ± 0.03	0.18 ± 0.01	1.9 ± 0.2	
Chlorpheniramine[b]				0.066 ± 0.008

[a] Values are the means ± standard errors of the mean for two independent experiments, each in duplicate.
[b] Cocaine and chlorpheniramine were used as positive controls.

unique inhibitory activity of verapamil against QN in the 106/1^{76I} line was also observed with KF-A6. Against all parasite lines, the chemosensitization patterns of KF-A6 on CQ and QN mirrored that of verapamil.

Effects of acridone derivatives on inhibition of neurotransmitter uptake activity and binding to histamine receptor H1. The clinical utility of tricyclic antidepressants and antihistamine compounds as antimalarial chemosensitizers is limited by adverse effects at the high doses required for chemosensitizing efficacy. Since the antidepressants are believed to inhibit neurotransmitter uptake by the biogenic amine transporters (17) and the antihistamines act by competing with histamine for H1 receptor sites on effector cells (31), the acridone chemosensitizers were evaluated with well-established assays for their effects on the uptake of biogenic amines by recombinant hDAT, hSERT, and hNET transporters stably expressed in HEK 293 cells, as well as their effects on binding to recombinant human histamine H1 receptor from CHO cells labeled with [^{3}H]pyrilamine.

As shown in Table 5, the well-studied chemosensitizers tricyclic antidepressants desipramine and imipramine showed high specificity for blocking the uptake of serotonin and norepinephrine. In contrast, acridone derivatives showed essentially no effect on all three transporters.

Chlorpheniramine, an H1 antagonist antihistamine, was a potent competitor for the specific binding of [^{3}H]pyrilamine to human histamine H1 receptor, with an IC_{50} (from the competition curve) of 66 nM, whereas KF-A6 at an effective chemosensitizing concentration, was substantially weaker, with a IC_{50} of 3.6 μM.

DISCUSSION

The combined results demonstrate that the novel 10-N-substituted acridones, with the rigid aromatic tricyclic ring system, represent efficacious chemosensitization pharmacophores. The acridone derivatives, with the side chain bridged three or more carbon atoms apart between ring nitrogen and terminal nitrogen, possess marked ability to enhance the efficacy of CQ and its major metabolite, DCQ, as well as other quinoline antimalarials, such as QN, against MDR *P. falciparum*. Moreover, the acridone constructs demonstrate significant synergy with CQ in MDR parasites by FIC isobologram analysis.

The discussion of possible mechanisms of action for acridones and chemosensitizers in general to reverse quinoline resistance undoubtedly needs to start with the antimalarial mode of action of the quinolines and the mechanism of resistance to these drugs in malaria parasites. Unfortunately, despite years of use and study, both mechanisms remain unresolved. However, it is widely accepted that the site of action for CQ is the acidic food vacuole, where CQ accumulates via acid trapping. It has also been proposed that CQ inhibits the formation of hemozoin, the detoxification product formed upon hemoglobin degradation, leading to heme-induced parasite death. CQ resistance appears to be the result of reduced drug accumulation in the food vacuole, leading to decreased drug access to the target heme (19), although various models for the explanation of this phenomenon are still under debate (12, 21, 44, 45). While specific *P. falciparum* quinoline-resistant phenotypes are likely to be multigenic in origin (15), the most important determinant of CQR is conferred by point mutations in a putative transporter protein, PfCRT, located in the food vacuole membrane of the parasite, with one amino acid change at position 76 playing a critical role (14, 15, 18, 60). Mutations within PfCRT, particularly changes from a charged amino acid residue (lysine, K76) to an uncharged residue (such as threonine [76T], asparagine [76N], or isoleucine [76I]), seem to be important not only in the acquisition of resistance to quinoline antimalarials (e.g., by allowing efflux of diprotic CQ), but also in the mechanism of resistance reversal actions for chemosensitizers (14, 15, 18, 37, 61, 62).

Similarly to the proposed mechanism of action for verapamil, the acridone chemosensitizers may compete for the CQ binding site in PfCRT and reduce CQ efflux from the food vacuole by a charge-repulsion effect, replacing the lost positive charge of K76 with a protonated amino group. Presumably, the weakly basic acridone derivatives (such as KF-A4, KF-A5, and KF-A6) diffuse across biological membranes of the parasite in the unprotonated form, and once they enter the acidic food vacuole, they are rapidly trapped via weak base properties (acid trapping) as ionized forms. In the acidic vacuole, these acridones may alter the pH of the environment or interfere with the efflux of CQ, restoring the efficacy of CQ and other quinoline antimalarials. While the effect of position 76 mutations on the chemosensitizing activity of KF-A6 offers evidence

for a direct interaction with the PfCRT protein, the absence of chemosensitizing activity from KF-A6-Cl (lacking the protonable terminal tertiary amine group) further supports our hypothesis. Given the ease of chemical synthesis of 10-N-substituted acridones, additional studies to explore the relationship of structural modifications of acridone derivatives and their chemosensitization activities, most importantly in PfCRT mutants, could help us to further understand the molecular basis of chemosensitizers and possibly provide greater insight into the resistance mechanism of quinoline antimalarials.

A major problem with existing antimalarial chemosensitizers is their neurological side effects at the high doses required to achieve optimal chemosensitization effects. The dose-limiting toxic effects of the antidepressants and antihistamines are mediated by their modes of action, which involve the inhibition of neurotransmitter uptake by the biogenic amine transporters and competition with histamine for receptor sites on effector cells, respectively (17, 52, 56). We believe it is important to show that novel tricyclic chemosensitizers without these effects are possible, and therefore, we have adopted a screening model to assess the effects of drugs on the uptake of biogenic amines by recombinant hDAT, hSERT, and hNET transporters, as well as on binding to human histamine receptor H1. These well-established high-throughput assays represent a valuable aid for the assessment of chemosensitizers, as well as for down-selection of candidate compounds in antimalarial-drug development. The absence of effect on the uptake of biogenic amines or binding to the histamine receptor distinguishes these acridones from other known chemosensitizers (i.e., desipramine, imipramine, and chlorpheniramine) and demonstrates that chemosensitization potency is achievable without the undesirable collateral sequelae.

Chemosensitizers clearly remain of great importance in mechanistic studies, but the challenges of putting them into clinical use reach far beyond safety obstacles. The idea of restoring CQ usefulness is still enticing for the following reasons: (i) CQ remains the least expensive and most readily available antimalarial in Africa (22), (ii) CQ is safe to use in pregnant women and children, and (iii) withdrawal of CQ resistance in some areas raises the possibility of a CQ comeback (38). However, challenges remain, as a chemosensitizer would provide assistance to CQ only in combination therapy, and hence, a "cocktail" with an additional partner drug may be required, making it less efficient, more costly, and thus impractical for malaria treatment where resources are greatly limited. In recognition of this predicament and the need for combination therapy in the age of increasing drug resistance, studies are under way to investigate the feasibility of combining chemosensitization utility with intrinsic antimalarial potency in the same acridone molecule.

ACKNOWLEDGMENTS

We thank Patricia Pauletti from the Department of Organic Chemistry, São Paulo State University, Araraquara, Brazil, for her assistance in the preparation of KF-A6 and Dennis Kyle from the Walter Reed Army Institute of Research, Silver Spring, MD, for the generous gift of DCQ. We are grateful to the Malaria Research and Reference Resource Center for supplying *P. falciparum* parasites D6 and Dd2.

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