Optimization of Xanthenes for Antimalarial Activity: the 3,6-Bis- ω -Diethylaminoalkoxyxanthone Series

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Hydroxyxanthenes have been identified as novel antimalarial agents. The compounds are believed to exert their activity by complexation to heme and inhibition of hemozoin formation. Modification of the xanthone structure was pursued to improve their antimalarial activity. Attachment of R-groups bearing protonatable nitrogen atoms was conducted to enhance heme affinity through ionic interactions with the propionate side chains of the metalloporphyrin and to facilitate drug accumulation in the parasite food vacuole. A series of 3,6-bis- ω -diethylaminoalkoxyxanthenes with side chains ranging from 2 to 8 carbon atoms were prepared and evaluated. Measurement of heme affinity for each of the derivatives revealed a strong correlation ($R^2 = 0.97$) between affinity and antimalarial potency. The two most active compounds in the series contained 5- and 6-carbon side chains and exhibited low nanomolar 50% inhibitory concentration (IC_{50}) values against strains of chloroquine-susceptible and multidrug-resistant *Plasmodium falciparum* in vitro. Both of these xanthenes exhibit stronger heme affinity (8.26×10^5 and 9.02×10^5 M⁻¹, respectively) than either chloroquine or quinine under similar conditions and appear to complex heme in a unique manner.

Malaria represents the most deadly parasitic human disease, despite countless efforts to eradicate or control it. Each year, it threatens roughly 40% of the world's population, infects over 200 million people, and claims 2 million lives—primarily children under 5 years of age (32, 36). To make matters worse, treatment of malaria is becoming increasingly more difficult due to the emergence of multidrug-resistant strains of *Plasmodium falciparum*, causative agent of the most severe form of the disease (32). As a result, there is a pressing need to develop novel antimalarial agents.

The *Plasmodium* parasite infects erythrocytes and digests hemoglobin within an acidic food vacuole for salvage of critically needed amino acids (21). This proteolytic process results in the release of toxic heme, which is sequestered into an insoluble nontoxic substance known as hemozoin, or more commonly as “malaria pigment” (1, 22, 27). The classical antimalarial quinolines, quinine and chloroquine, are believed to act via complexation to heme in a manner that perturbs hemozoin formation (7, 10, 23, 26, 30).

Hydroxyxanthenes have been identified by us as a novel class of antimalarial compounds with activity against multidrug-resistant *Plasmodium* parasites (34, 35). We have demonstrated that selected hydroxyxanthenes form soluble complexes with heme and prevent the precipitation of heme in aqueous solution under the mildly acidic conditions of the digestive vacuole at pH 5.2 (14). In a recent study of the interaction between heme and 4,5-dihydroxyxanthone, a model that features carbonyl-iron coordination, π - π stacking of the coplanar aromatic molecules, and hydrogen bonding between the drug's hydroxyl

substituents and the propionate side chains of heme was found to be consistent with the proton nuclear magnetic resonance (NMR) data (17). The molecular model of the drug-heme complex led us to speculate that positioning of protonatable amines in the lower portion of the xanthone nucleus (i.e., positions 3 through 6) would enhance affinity for free heme and improve the antimalarial properties of the drug. The cationic amines would form an ionic bidentate interaction with the target heme carboxylates. Herein we describe the evaluation of a second generation of compounds containing this design feature, the 3,6-bis- ω -diethylamino-alkoxyxanthenes, and provide clear evidence of their ability to target parasite heme metabolism in a unique manner.

MATERIALS AND METHODS

Materials. Heme chloride was purchased from Sigma Chemical Company (St. Louis, Mo.). [³H]ethanolamine was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, Mo.). Detailed methods for synthesis of the 3,6-bis- ω -diethylaminoalkoxyxanthenes will be published elsewhere. The final products were subjected to elemental analysis, ¹H-NMR spectroscopy, and mass spectrometry to ensure identity and purity to >99%.

Culture of *P. falciparum* and IC_{50} determinations. The chloroquine-susceptible clone D6 and multidrug-resistant clone W2 were obtained from Dennis E. Kyle of the Walter Reed Army Institute for Research (Silver Spring, Md.). The chloroquine-resistant strain F86 (16) was purchased from the American Type Culture Collection (Manassas, Va.). The parasites were cultured according to the method of Trager and Jensen (29), with minor modification. Cultures were maintained in fresh group A⁺ human erythrocytes; suspended at a 2% hematocrit in RPMI 1640 (pH 7.2) containing 10% fresh human serum, 3 g of glucose per liter, 45 μ g of hypoxanthine per ml, and 50 μ g of gentamicin per liter; and incubated at 37°C under a gas mixture of 5% O₂, 5% CO₂, and 90% N₂. Stock cultures were passaged every 3 to 4 days by transfer of infected erythrocytes to a flask containing complete medium and uninfected erythrocytes.

The in vitro antimalarial activity of each drug was determined by monitoring incorporation of [³H]ethanolamine (50 Ci/mmol) into parasite lipids in the presence and absence of drug, essentially as described by Elabbadi et al. (11) with slight variations. Xanthone derivatives were dissolved in ethanol to give a 10 mM stock solution and then diluted with medium to achieve the desired concentra-

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FIG. 1. Generalized structure of 3,6-bis- ω , N,N -diethylaminoalkoxyxanthenes.

tion. The final concentration of ethanol never exceeded 1% (vol/vol), which was found to be nontoxic to the parasites. The experiments were set up in duplicate in 96-well plates. Each well contained 180 μ l of complete medium with parasitized erythrocytes (0.2% parasitemia); wells containing unparasitized erythrocytes served as background controls. Twenty-microliter dilutions of each drug ($10\times$ final concentration) were added across the plate to achieve the final concentrations in the range of 10^{-9} to 10^{-4} M and including a drug-free reference control.

[^3H]ethanolamine (50 Ci/mmol [1 μ Ci per 20 μ l of medium]) was added after 48 h, and the experiments were terminated after 72 h of incubation by collecting the cells onto glass fiber filters with a semiautomated Tomtec (Orange, Conn.) 96-well plate harvester. [^3H]ethanolamine uptake was quantitated by scintillation counting of the filters with a Wallac (Gaithersburg, Md.) 1205 Betaplate counter. The concentration of a drug giving 50% inhibition of label incorporation (IC_{50}) relative to drug-free controls was calculated by nonlinear regression analysis of the semilogarithmic dose-response curve. Each IC_{50} value ($\pm$ standard deviation) was derived from the average of at least three separate experiments, each performed in duplicate.

Interaction of xanthenes with heme. Association constants for heme-xanthone complexes were determined by spectrophotometric titration with a Varian-Cary 3E spectrophotometer with 1-cm quartz cuvettes and a thermostatted cell holder.

All titrations were conducted in 20 mM phosphate buffer at pH 7.0 and 25°C. An aliquot of a 10 mM stock solution of heme in 0.01 N NaOH was diluted to 10 μ M in phosphate buffer. Titrations with each of the tested drugs were performed by successive addition of aliquots of a 1 mM stock solution to the 10 μ M heme solution; pH was monitored throughout the procedure and was found to remain unchanged. All UV-visible spectral data were converted from the SPC (Varian-Cary) data format to Excel (Microsoft) data with Gram 386 software (Galactic Industries) and then processed with Excel software (Microsoft). Absorbance readings and concentrations were corrected for dilution effects, including all plotted spectra. The resulting titration curves were analyzed with a Hill plot (3) and nonlinear curve-fitting methods (4).

RESULTS

Antimalarial activities. The 3,6-bis- ω , N,N -diethylaminoalkoxyxanthone series (Fig. 1) contains R-groups with nitrogen atoms that become protonated under the acidic conditions of the food vacuole and are designed to provide a strong ionic association with heme's propionate groups. Because the strength of such an interaction is dependent on both bond length and the dielectric constant of the aqueous milieu of the vacuole (the latter being unknown to us), we prepared the straight-chain disubstituted series from C2 to C8 (except for C7), each with terminal diethylamino groups. The IC_{50} values for this xanthone series in our 72-h assay range from 0.075 to 2.2 μ M, as listed in Table 1. Within the series, 3,6-bis- ϵ -(N,N -diethylamino)amyloxyxanthone (C5) and 3,6-bis- ζ -(N,N -diethylamino)-hexyloxyxanthone (C6) show the highest potency

TABLE 1. In vitro antimalarial activity of selected nitrogenated xanthenes against D6, W2, and F86 strains of *P. falciparum* and apparent binding affinity (K_a) for heme in aqueous solution^a

Chemical name	Structure	IC_{50} (μM)			K_a (10^5 M^{-1})
		D6	W2	F86	
3,6-Bis- β -(N,N -diethylamino)-ethoxy xanthone, C2		2.2 $\pm$ 0.5			3.44 $\pm$ 1.18
3,6-Bis- γ -(N,N -diethylamino)-propoxy xanthone, C3		1.5 $\pm$ 0.7			3.84 $\pm$ 1.71
3,6-Bis- δ -(N,N -diethylamino)-butoxy xanthone, C4		0.65 $\pm$ 0.08			6.95 $\pm$ 1.92
3,6-Bis- ϵ -(N,N -diethylamino)-amyloxy xanthone, C5		0.10 $\pm$ 0.05	0.12 $\pm$ 0.07	0.11 $\pm$ 0.06	8.26 $\pm$ 0.96
3,6-Bis- ζ -(N,N -diethylamino)-hexyloxy xanthone, C6		0.07 $\pm$ 0.02	0.07 $\pm$ 0.03	0.07 $\pm$ 0.02	9.02 $\pm$ 1.23
3,6-Bis- θ -(N,N -diethylamino)-octyloxy xanthone, C8		0.43 $\pm$ 0.03			7.97 $\pm$ 2.10

^a IC_{50} , concentration of compound that reduced parasite growth (percent incorporation of radiolabeled ethanolamine) by 50% relative to drug-free controls. IC_{50} and K_a values are means $\pm$ standard deviations of multiple independent determinations.

FIG. 2. Effect of carbon chain length on the antimalarial activity of 3,6-bis- ω -*N*, *N*-diethylaminoalkoxyxanthenes.

against the D6 clone of *P. falciparum*, with IC₅₀ values of 0.1 and 0.075 μ M, respectively. The remarkable correlation between the length of the side chain and antimalarial potency is shown in Fig. 2. In contrast to our findings with this xanthone series, consider that chloroquine analogs, varying in side chain length from 2 to 12 carbon atoms, exhibit nearly identical IC₅₀s against drug-sensitive *P. falciparum* (6).

We also determined IC₅₀ values for C5 and C6 against a chloroquine-resistant strain (F86) and the multidrug-resistant strain (W2) of *P. falciparum*. We observed that the xanthone derivatives were equipotent against all three strains irrespective of their drug resistance profiles (Table 1), thereby demonstrating that the mechanism of action is unique and that xanthone-based antimalarials circumvent mechanisms of multidrug resistance. These findings stimulated us to investigate the possibility of a correlation between the heme binding affinity of xanthenes and their antimalarial potency.

Interaction of 3,6-bis- ω -(*N,N*-diethylamino)alkoxyxanthenes with heme. A solution of heme at pH 7.0 showed a broad peak at 386 nm (Fig. 3, top trace), indicating that the dimer form of heme predominates under our in vitro conditions, as expected (15, 33). In the aqueous solution under study, it is presumed that heme exists as a mixture of μ -oxo dimers (2) and β -hematin dimers (8, 18, 22), the latter representing a key structural intermediate in the aggregation of dimeric heme units to form hemozoin. Since the true nature of heme in aqueous solution remains controversial, we make no attempt to distinguish between these two structural forms of heme aggregate and merely refer to heme dimer in general terms. The concentrated stock solutions were made freshly and diluted just before use; the titration measurements were completed within 5 min after each dilution. A control of diluted heme solution (10 μ M) was tested spectroscopically before and after the measurement; the drop in the Soret band height proved to be negligible during this 5-min period. Furthermore, a dilution test showed that the Beer-Lambert law (28) was obeyed up to the concentration of 20 μ M heme, indicating that

further aggregation did not occur during the test period in the control heme solution (33).

Titration with increasing amounts of C5 into the heme solution clearly caused spectroscopic changes, as shown in Fig. 3, producing spectra with a well-defined isosbestic point in the Soret range, reflecting a reduction in the heme Soret molar absorptivity and a shift of the Soret band to longer wavelengths. The loss of Soret band intensity may result from the formation of π - π complexes (25) or aggregation and precipitation of heme (33). To investigate both possibilities, a dilution test was performed with a solution containing C5 and heme in

FIG. 3. Spectroscopic changes observed in the Soret region of heme on addition of C5 at pH 7.0 and 25°C in 20 mM phosphate buffer. A representative number of spectral scans are shown. All spectra were corrected for dilution.

FIG. 4. Variation in absorbance of heme (10 μ M) at 386 nm as a function of C5 concentration. The solid line is a best fit to model 1, as represented by equation 2 in the text. The titration was conducted at pH 7.0 and 25°C. (Inset) Comparison of the various models of C5 interacting with heme as described in the text.

a molar ratio of 10 to 1. Dilution of this sample was found to obey the Beer-Lambert law in the concentration range of 1 to 50 μ M heme. This observation indicates that further heme aggregation and/or precipitation does not occur under our test conditions. Therefore, based on the previous evidence of UV-visible and $^1\text{H-NMR}$ spectroscopy of hydroxyxanthone-heme mixtures (17), the most reasonable explanation for the observed spectral changes is that they do indeed represent C5-heme association.

The interaction of heme with C5 was monitored in the Soret region of the UV-visible spectrum. The absorbance changes at 386 nm as a function of drug concentration are presented in Fig. 4. The mode of interaction between C5 and heme was investigated in detail. Five different models were evaluated as to their ability to fit the data in an effort to obtain the most accurate possible binding isotherm and equilibrium binding constant. In considering each of the following models, Hm_2 represents the concentration of heme dimer and C represents the concentration of the antimalarial agent, C5. The value of $[\text{C}]_{\text{free}}$ was determined numerically by Newton's method by using the polynomial forms of the equations described in each case (4, 19).

Model 1. The simplest model assumes a single C5 molecule in association with a single heme dimer. The reaction is represented by equation 1:

The change in absorbance as a function of free C5 concentration is given by equation 2:

$$A = \frac{A_0 + A_\infty K_a [\text{C}]_{\text{free}}}{1 + K_a [\text{C}]_{\text{free}}} \quad (2)$$

where A_0 is the absorbance without C5 added, A_∞ is the absorbance for the heme-C5 complex, and K_a is the apparent binding constant for the interaction. The observed experimental absorbance data were fit as a function of C5 concentration to equation 2 by the nonlinear least-squares method with A_0 , A_∞ , and K_a as variables. As shown in Fig. 4, the experimental data conform very closely to the model across the entire region of titration. The apparent binding constant (K_a) derived from the fitting is $8.26 \times 10^5 \text{ M}^{-1}$ (Table 1). The interaction was also evaluated by the Hill method, as shown in Fig. 5. The Hill plot gave a reasonably straight line with a slope near unity (0.995), consistent with the binding isotherm (i.e., one C5 molecule in association with one Hm_2) and indicative of the non-cooperative nature of Hm_2 -C5 binding. The apparent binding constant derived from the Hill plot ($8.15 \times 10^5 \text{ M}^{-1}$) is consistent with the result obtained by the nonlinear least-squares fitting method.

Despite the excellent fitting for model 1, four additional models were examined in order to evaluate other possible modes of interaction.

Model 2. Two C5 molecules bind simultaneously to one heme dimer, as given in equation 3:

Model 3. Two C5 molecules bind to one heme dimer unit in stepwise fashion, as represented by equation 4:

FIG. 5. Hill plot of heme-C5 association at 386 nm, pH 7.0, and 25°C. The slope of the line is 0.99, and the intercept (the log of the apparent binding constant) is 5.91.

Model 4. A single C5 molecule binds simultaneously to two heme dimer units as given in equation 5:

Model 5. A single C5 molecule binds sequentially to two heme dimer units, as represented by equation 6:

The mathematical expressions representing the dependence of absorbance on free ligand concentration according to complexation models 2 to 5, respectively, have been published previously (9, 19). We adapted these equations to compare the fit of the C5 data to models 2 through 5 (inset of Fig. 4). While model 2 conformed closely to the experimental data for low concentrations of the drug, it did not provide a good match at intermediate and high concentrations of C5. Model 3 yielded an inferior fit of the experimental data through the graphical region of intermediate to high drug concentrations. The poor fit of both models 2 and 3 excludes the possibility of two drug molecules interacting with heme as a “sandwich” with a heme

dimer in the center. Attempts to optimize these variables freely in model 4 proved unsuccessful, and the model fit the data poorly across the entire range of concentrations, even with a manual grid search. While we observed that the data fit model 5 reasonably well with derived $K_{a1} = 7.96 \times 10^5 \text{ M}^{-1}$ and $K_{a2} = 1.59 \text{ M}^{-1}$, the K_{a1} value is very close to the K_a value derived from model 1 and the K_{a2} value is so small as to be negligible (not shown). This result confirms the superior fit for model 1, which assumes that a single C5 molecule complexes to one heme dimer unit under our experimental conditions.

To obtain the apparent binding constants for the other xanthenes in this series, the spectroscopic studies were performed with each drug as described for C5, and the data were processed and analyzed in the same manner. In each case, it was observed that the xanthone derivative complexes to heme with a stoichiometry of one drug molecule to one heme dimer. The apparent binding constant for each drug-heme binding interaction is listed in Table 1. As shown, the C5 and C6 derivatives bound more avidly to heme than did the other xanthenes in the series.

Correlation of heme affinity to antimalarial potency. As shown in Fig. 6, there is a nearly linear correlation ($R^2 = 0.97$) between heme binding affinity and antimalarial potency for the 3,6-bis- ω -diethylaminoalkoxyxanthenes. The correlation provides compelling evidence that high binding affinity to heme, as demonstrated in C5 and C6, is a critical determinant in their antimalarial potency.

DISCUSSION

The *Plasmodium* digestive vacuole is an acidic compartment central to the metabolism of the parasite. It is where hemoglobin is degraded by specific proteases to provide amino acids

FIG. 6. Correlation of antimalarial potency (IC_{50}) versus heme affinity (K_a) for the 3,6-bis- ω - N,N -diethylaminoalkoxyxanthenes.

for parasite survival (12, 21). This process liberates an enormous amount of heme, which is converted spontaneously into insoluble hemozoin (1, 22, 23, 27). Recent studies indicate that hemozoin is an ordered arrangement of dimeric heme units, linked together by reciprocal iron-propionate bonds (8, 22). The 4-aminoquinoline chloroquine accumulates in the vacuole and perturbs hemozoin formation (13, 37). While it is unclear how the process is affected to the detriment of the parasite, extensive studies have shown that chloroquine forms a stable complex with two μ -oxo heme dimers (7, 10, 23, 26, 30). Drug-induced accumulation of μ -oxo dimers may disrupt the normal lattice of reciprocal dimers, leading to structural disorganization and deterioration of the acidic vacuole. Chloroquine resistance appears to derive from a complex mosaic of mutations that encode altered proteins that perturb the normal disposition of the drug in resistant parasites in such a manner as to hinder chloroquine's access to the target molecule, heme. The resistance mechanism appears to be specific for chloroquine's diethylaminoisopentyl side chain (5, 20, 31).

Despite the emergence of quinoline resistance and the likelihood that this phenomenon stems from an alteration affecting the parasite food vacuole, enzymes and processes within this unique organelle still represent excellent drug targets (21, 24). Furthermore, since heme does not accumulate in the acidic compartments of mammalian cells and is an immutable product of a process that is central to parasite survival, free and dimeric heme remain legitimate targets for antimalarial drug design.

Our studies highlight the 3,6-bis- ω -(N,N -diethylaminoalkoxy)xanthenes, for which there is a clear correspondence between side chain length, heme affinity, and antimalarial potency. Such a correlation does not exist for side chain derivatives of chloroquine or quinine (6). The accumulated evidence points to heme as the primary target of xanthone action. C5 and C6, two lead compounds that emerged from this work, exhibited the greatest affinity for heme among the xanthenes

tested. These two compounds were over 1,000 times more potent than either 3,6-dihydroxyxanthone (14) or 3,6-bis-diethylaminoxanthone (not shown), and they were equally active against drug-resistant parasites. Compared to literature values, both C5 and C6 exhibited stronger heme affinities than either chloroquine or quinine (7) and appear to complex heme in a distinctly different manner. The two aminoquinolines bind to heme with a stoichiometry of between 4 and 8 heme units per molecule of drug (7), whereas C5 and C6 form stable complexes with 2 heme units, which we presume to be in the dimer state. At this time, we may only speculate that C5 and C6 bind to a heme dimer lacking planar symmetry, as was found in the case of 4,5-dihydroxyxanthone (17). If this is the case, the targeted dimer units may be intermediate forms of heme dimers linked together by reciprocal iron-propionate bonds, which are now known to be involved in the formation of hemozoin in the parasite food vacuole (22).

The improved potency of the lead compounds, C5 and C6, indicate that it is now appropriate to initiate in vivo testing in a murine malaria model. These studies are currently under way, and the results will be published in due course. Meanwhile, in this laboratory, research continues on the rational design and optimization of novel antimalarial agents that target immutable heme and hemozoin synthesis to achieve selective parasite killing.

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