

# Photoaffinity Labeling of Chloroquine-binding Proteins in *Plasmodium falciparum*\*

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A photoreactive analog of chloroquine, *N*-(4-(4-diethylamino-1-methylbutylamino)quinolin-6-yl)-4-azido-2-hydroxybenzamide (referred to as ASA-Q), has been synthesized and shown to mimic the action of chloroquine in possessing substantial antimalarial activity against a chloroquine-sensitive strain of *Plasmodium falciparum*. As for chloroquine, ASA-Q is less effective at killing drug-resistant strains of malaria, and the resistance can be modulated using the reagent verapamil. ASA-Q has been radiolabeled with Na<sup>125</sup>I and used as a photoaffinity probe for labeling chloroquine-binding proteins in malaria-infected erythrocytes. Two proteins have been identified with apparent molecular masses of 42 and 33 kDa in both chloroquine-sensitive and chloroquine-resistant strains of malaria. Photoaffinity labeling of the two proteins by iodo-ASA-Q was competitively inhibited by an excess of unlabeled chloroquine. The structurally related antimalarials amodiaquine and quinine also inhibited labeling of the two proteins, while verapamil and doxycyclin had no effect. We suggest that the two labeled proteins are the macromolecular targets of chloroquine action in malaria parasites.

Chloroquine is a very widely used antimalarial drug which was synthesized as an analog of quinine and which has remained a major chemotherapeutic agent for over 40 years. The popularity of chloroquine has been due to its high efficacy and the low risk of side effects when it is taken in the correct dosage. Unfortunately, the operational use of chloroquine has been severely hampered in recent years, due to the increasing incidence of chloroquine resistance.

Despite its widespread use, the mode of action of chloroquine, and of other quinoline drugs, is not completely understood. Chloroquine is a diprotic weak base that accumulates in the acidic food vacuole of the malaria parasite (Homewood *et al.*, 1972; Krogstad *et al.*, 1985). At therapeutic serum concentrations, the vacuolar concentration of chloroquine has been estimated to reach millimolar levels (Ginsberg and Geary, 1987). It has been suggested by some workers that accumulation may be enhanced by the action of a permease (Warhurst, 1986), or by binding of chloroquine to a "receptor" within the vacuole (Fitch, 1986), although the need to invoke a specific uptake mechanism has been disputed (Geary *et al.*, 1990).

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High levels of intravacuolar chloroquine are assumed to interfere with the parasite feeding mechanism, as parasites are only susceptible to chloroquine in the mature stages of the asexual life cycle when they actively digest hemoglobin to produce the malarial pigment hemozoin (Peters, 1970). A number of workers have suggested that chloroquine inhibits the action of proteins involved in hemoglobin digestion or heme disposal (see Ginsberg and Geary, 1987, for review); however, the precise target at the molecular level has not been unambiguously defined. Hemoglobin digestion by *Plasmodium falciparum* is an ordered metabolic pathway. The initial events are the endocytosis of hemoglobin from the host cytoplasm and transport to the food vacuole where the protein is degraded by a series of proteolytic enzymes (Vander Jagt *et al.*, 1987). The heme, which is released as a by-product of hemoglobin degradation, cannot be metabolized by the parasite and is instead detoxified by the activity of a putative heme polymerase (Slater and Cerami, 1992). Fitch (1972) originally proposed that chloroquine forms a complex with free heme which is directly lytic to the parasite; however this hypothesis is no longer favored due to a failure to demonstrate sufficiently high levels of free heme (Yayon *et al.*, 1985). Possible targets for chloroquine thus include proteins involved in the endocytic process, hemoglobin proteases, or, alternatively, the heme polymerase.

Over the last 30 years, chloroquine resistance has become an increasingly serious problem and is now observed in all countries where malaria is endemic. The biochemical basis of resistance is not completely understood; however it is clear that resistant parasites accumulate chloroquine less efficiently than sensitive isolates (Fitch, 1972; Yayon *et al.*, 1985). It has been suggested that the mechanism of chloroquine resistance has similarities with the multiple drug resistance phenotype of mammalian tumor cells, as verapamil, an agent which reverses multiple drug resistance of tumor cells, is also able to modulate chloroquine resistance (Krogstad *et al.*, 1987; Martin *et al.*, 1987).

If proteins involved in chloroquine action and resistance could be identified and characterized, a functional approach to the design of novel antimalarials would become a possibility. In this study, we have synthesized a photoreactive analog of chloroquine and used it to identify two chloroquine-binding proteins which may represent the targets of chloroquine action in *P. falciparum*.

## MATERIALS AND METHODS

*Synthesis of N*-(4-(4-Diethylamino-1-methylbutylamino)quinolin-6-yl)-4-azido-2-hydroxybenzamide (ASA-Q, Compound 5)—ASA-Q was prepared by a multistep synthesis as illustrated in Fig. 1. 6-Nitro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid was prepared according to

<sup>1</sup> The abbreviations used are: ASA-Q, *N*-(4-(4-diethylamino-1-methylbutylamino)quinolin-6-yl)-4-azido-2-hydroxybenzamide; TLC, thin layer chromatography; PAGE, polyacrylamide gel electrophoresis.

Riegel *et al.* (1946), decarboxylated by way of the silver salt (Baker *et al.*, 1946), and converted to compound 1, melting point 140 °C (from light petroleum (boiling point 90–110 °C)) (Baker *et al.*, 1946; 141–141.5 °C).

A mixture of compound 1 (1.3 g) and 5-diethylamino-2-pentylamine (5 ml) was heated at 100 °C for 5 h. The mixture was then partitioned between ether and 5% sodium hydroxide solution, and the ether extract was washed five times with water, dried (MgSO<sub>4</sub>), and the solvent removed to give the nitroamine (compound 2) as an oil (1.9 g). <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 1.1, t, *J* = 7 Hz, CH<sub>2</sub>CH<sub>3</sub>; 1.4 d, *J* = 6 Hz, CHCH<sub>3</sub>; 1.8, br s, (CH<sub>2</sub>)<sub>2</sub>; 2.4–2.75, m, NCH<sub>2</sub> + CH<sub>2</sub>CH<sub>3</sub>; 3.6–4.0, m, CH; 6.35, d, *J* = 8 Hz, NH; 6.6, d, *J* = 6 Hz, H-3; 8.05, d, *J* = 9 Hz, H-8; 8.4, dd, *J* = 9.2 Hz, H-7; 8.7, d, *J* = 6 Hz, H-2; 8.95, d, *J* = 2 Hz, H-5.

A mixture of compound 2 (1.9 g) and 10% palladium/charcoal (0.5 g) in ethanol (40 ml) was treated with hydrazine hydrate (5 ml) and then refluxed for 1 h. The catalyst was filtered off and the solvent evaporated. The residue was dissolved in ether, separated from a small amount of hydrazine, and the ether extract was washed with a little cold water, dried, and the solvent evaporated to give 6-amino-4-(4-diethylamino-1-methylbutyl)aminoquinoline, compound 3, as a brown oil (1.3 g, 75%), sufficiently pure for the next reaction. <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ 1.0, t, *J* = 7 Hz, CH<sub>2</sub>CH<sub>3</sub>; 1.3, d, *J* = 6 Hz, CHCH<sub>3</sub>; 1.6, br s, (CH<sub>2</sub>)<sub>2</sub>; 2.3–2.7, m, NCH<sub>2</sub> + CH<sub>2</sub>CH<sub>3</sub>; 3.6–4.0, m, CH + NH<sub>2</sub>; 4.7, d, *J* = 8 Hz, NH; 6.35, d, *J* = 6 Hz, H-3; 6.8, d, *J* = 2 Hz, H-5; 7.05, dd, *J* = 9.2 Hz, H-7; 7.8, d, *J* = 9 Hz, H-8; 8.35, d, *J* = 6 Hz, H-2.

A solution of azidosalicylic acid (compound 4, 0.129 g) (Husain *et al.*, 1983) and 1,1'-carbonyldiimidazole (0.125 g) in dioxan (3 ml) was taken to the boiling point for 10 min and then left at room temperature for 1 h. To this was added compound 3 (0.1 g) in dioxan (0.5 ml), and the solution was warmed for 10 min and then left overnight at room temperature. The solid which separated was filtered and washed with dioxane to give the fawn ASA-Q product (compound 5, 0.06 g, 39%), melting point 192–195 °C (dec) (from dioxane). TLC (silica, 1:1 methanol/chloroform with 1% ammonium hydroxide) gave *R*<sub>f</sub> 0.4 (spot went brown when irradiated briefly with 366 nm light). <sup>1</sup>H NMR (300 MHz, (CD<sub>3</sub>)<sub>2</sub>SO) δ 1.0, t, *J* = 7 Hz, CH<sub>2</sub>CH<sub>3</sub>; 1.25, d, *J* = 6 Hz, CHCH<sub>3</sub>; 1.6 br s, (CH<sub>2</sub>)<sub>2</sub>; 2.6–2.8, m, NCH<sub>2</sub> + CH<sub>2</sub>CH<sub>3</sub>; 3.8, br s, CH; 6.2, dd, *J* = 7.5, 1.5 Hz, H-5'; 6.3, d, *J* = 1.5 Hz, H-3'; 6.55, d, *J* = 5 Hz, H-3; 7.15, br d, *J* = 6 Hz, NH; 7.75, d, *J* = 8 Hz, H-8; 7.9, d, *J* = 7.5 Hz, H-6'; 8.2, dd, *J* = 8 Hz, H-7; 8.3, m, H-2,5. Analysis. Found: C, 62.8; H, 6.6; N, 20.8. C<sub>25</sub>H<sub>31</sub>N<sub>7</sub>O<sub>2</sub>·H<sub>2</sub>O requires C, 62.6; H, 6.9; N, 20.5%.

**Assessment of Antimalarial Activity of ASA-Q**—Malaria parasites were continuously cultured as described by Trager and Jensen (1976). *P. falciparum* FAC8 (Biggs *et al.*, 1989) is a chloroquine-resistant clone derived from another cloned line ITG2F6 (gift of L. Miller). Isolate 3D7 is a chloroquine-sensitive strain of *P. falciparum* (Foote *et al.*, 1989). Malaria parasites were plated at ~1% parasitemia (2% hematocrit), in 96-well trays in the presence of different concentrations of chloroquine or ASA-Q. Experiments with ASA-Q were conducted under reduced light. Parasites were incubated for 4 days, with daily replacement of the drug-supplemented medium. Growth curves were obtained in duplicate as described by Barnes *et al.* (1992), and the concentration of drug required to produce 50% inhibition of growth (IC<sub>50</sub>) was determined.

**Preparation of Carrier-free Iodo-ASA-Q**—The 2-hydroxyl function in *p*-azidosalicylate allows the introduction of a radiolabel in the aromatic ring by iodination with Na<sup>125</sup>I (Ji *et al.*, 1985). The iodination reaction was carried out under reduced light. ASA-Q was dissolved in dimethyl formamide (60 μl, 10 nmol) and mixed with 20 μl of carrier-free Na<sup>125</sup>I (1 mCi, 0.56 nmol, Amersham) and 20 μl of chloramine T (10 nmol) in 1 M K<sub>2</sub>HPO<sub>4</sub>, pH 7.4. After 3 min at room temperature, the reaction was quenched with 10 μl of 10% sodium metabisulfite solution and applied to a C<sub>18</sub> cartridge (Sep Pack, Waters). After washing the column with 20 ml of water to remove unreacted iodine, the bound material was eluted with 30 ml of methanol. The methanol was removed by rotary evaporation, and the product was purified on Silica 60 TLC (Merck) developed using acetone/methanol (2:1, v/v). Radiolabeled ASA-Q was visualized by autoradiography. Two separate radiolabeled products were detected corresponding to the di-iodinated ASA-Q (*R*<sub>f</sub> = 0.12) and monoiodinated (*R*<sub>f</sub> = 0.08) products. Both products were well separated from the unlabeled ASA-Q (*R*<sub>f</sub> = 0.04). The silica gel containing each of the iodinated products was collected and the products recovered by flash chromatography using chloroform/methanol/NH<sub>4</sub>OH (1:1:0.1, v/v/v) as eluant. After evaporation of the solvent, the product was redissolved in methanol, centrifuged to remove any remaining silica particles, and stored at –20 °C until use. The radiolabeled compounds were authenticated by comigration with unlabeled iodo-ASA-Q on analytical TLC developed with either acetone/methanol (2:1, v/v) or chloroform/methanol/ammonium hydroxide (9:1:0.1, v/v/v). The total yield was about 10%

with a theoretical specific activity of 1.78 mCi/nmol for [monoiodo]ASA-Q and 3.57 mCi/nmol for [diiodo]ASA-Q. Two separate preparations of carrier-free iodo-ASA-Q were used in the experiments presented in this study; the first preparation contained a mixture of mono- and di-iodinated ASA-Q and is referred to as iodo-ASA-Q. Later experiments were performed using highly purified samples of [monoiodo]ASA-Q and [diiodo]ASA-Q.

**Photoactivated Labeling of Malarial Proteins with Iodonated-ASA-Q**—Enrichment for erythrocytes infected with mature stage malaria parasites was achieved using a procedure based on the method of Aley *et al.* (1986). Experiments were performed using either a mixture of [monoiodo]ASA-Q (0.18 pmol) and [diiodo]ASA-Q (0.04 pmol), or highly purified [monoiodo]ASA-Q (0.13 pmol) or [diiodo]ASA-Q (0.07 pmol). Iodinated ASA-Q (0.5–1 μl) was added from a stock in dimethyl sulfoxide to 50 μl of erythrocytes infected with mature stage parasites (approximately 5% hematocrit) and incubated for 15 min at 37 °C, in reduced light. The samples were then irradiated with long wave UV light (365 nm, 20 °C) to induce photoactivation. Incorporation of radiolabel was found to reach a plateau after approximately 10 min of exposure to UV light; a 15-min illumination period was thus used in all experiments. An equal volume of SDS-PAGE sample buffer containing 5% β-mercaptoethanol (Laemmli, 1970) was added immediately after photoactivation. The samples were analyzed by SDS-polyacrylamide gel electrophoresis, and radiolabeled proteins were visualized by phosphorimager analysis (Molecular Dynamics). In some experiments, infected erythrocytes were solubilized in 0.5% Triton X-100 in a buffer comprising 150 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 8, with or without 1 mM β-mercaptoethanol. For competition experiments, unlabeled chloroquine, amodiaquine, verapamil, quinine, and doxycyclin (all from Sigma) were added from concentrated stocks in aqueous solution. Mefloquine (gift from Hoffmann-La Roche) was dissolved in ethanol/water (1:1, v/v) and salicylamide (Aldrich) in dimethyl sulfoxide. Final levels of organic solvent were less than 1% of the total volume. In all cases the competing drugs were incubated with the infected erythrocytes for 15 min prior to the addition of iodinated ASA-Q.

## RESULTS

**Synthesis of ASA-Q**—A photoreactive analog of chloroquine was prepared that incorporates an azidosalicylate moiety at the 6-position on the quinoline ring and which lacks the chlorine in the 7-position (Fig. 1). This analog was designed to have similar *pK<sub>a</sub>* values to chloroquine, so that it might be expected to accumulate in the same acidic organelles within the parasite. To determine if the synthetic chloroquine analog ASA-Q was suitable as a reagent for identifying chloroquine-interacting proteins, we analyzed its pharmacological properties compared with those of chloroquine itself.

**Assessment of Antimalarial Activity of ASA-Q**—The capacity of ASA-Q to arrest the growth of *P. falciparum* *in vitro* was determined for both chloroquine-sensitive and chloroquine-resistant parasites. As shown in Fig. 2*b*, ASA-Q was a potent inhibitor of the growth of chloroquine-sensitive *P. falciparum* (3D7); the IC<sub>50</sub> value for inhibition of growth was 18 ng/ml. By comparison, chloroquine inhibited the 3D7 growth with an IC<sub>50</sub> value of 11 ng/ml (Fig. 2*c*). FAC8, a chloroquine-resistant *P. falciparum* clone showed resistance to both chloroquine and ASA-Q (Fig. 2, *a* and *c*). The IC<sub>50</sub> values for inhibition of FAC8 by chloroquine and ASA-Q were 50 and 34 ng/ml, respectively. It has previously been demonstrated that resistance of FAC8 to chloroquine is apparently reversed in the presence of verapamil (Barnes *et al.*, 1992). In this study, we found that apparent reversal of the resistance of FAC8 to ASA-Q was achieved in the presence of 1 μg/ml verapamil (Fig. 2*a*; IC<sub>50</sub> for ASA-Q = 17 ng/ml). At this concentration, verapamil had no effect on the IC<sub>50</sub> for inhibition of growth of 3D7 by ASA-Q (Fig. 2*b*). The similar antimalarial activities of ASA-Q and chloroquine suggests that ASA-Q is a suitable photoaffinity ligand for labeling proteins involved in the mechanism of chloroquine action.

**Photoaffinity Labeling of Chloroquine-binding Proteins with Iodo-ASA-Q**—As chloroquine is thought to be most active against the trophozoite stage of *P. falciparum* (Zhang *et al.*, 1986), erythrocytes infected with mature stages of the chloro-

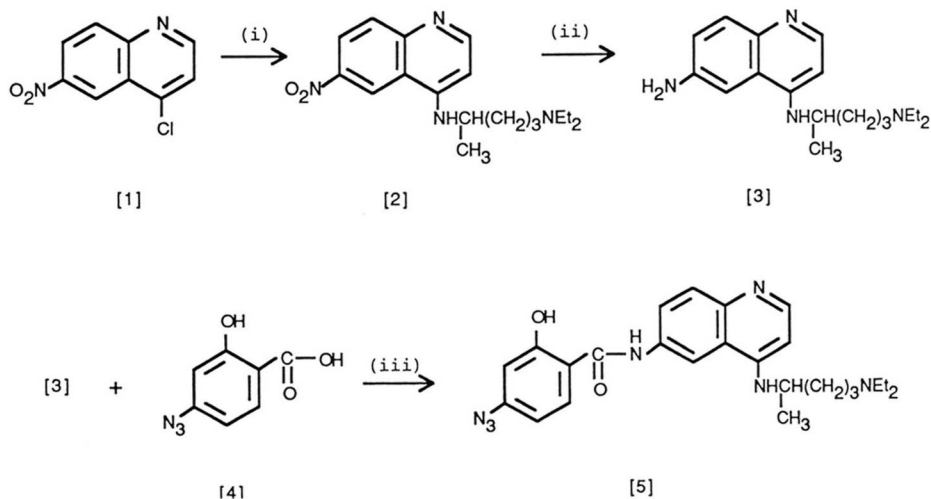


FIG. 1. **Synthesis of a photoaffinity ligand for chloroquine-binding proteins.** ASA-Q (compound 5) was synthesized as described under "Materials and Methods."  $\text{NH}_2\text{CH}(\text{CH}_3)(\text{CH}_2)_3\text{NEt}_2$ , heat (i);  $\text{N}_2\text{H}_4/\text{Pd/C}$  (ii); 1,1'-carbonyldiimidazole (iii).

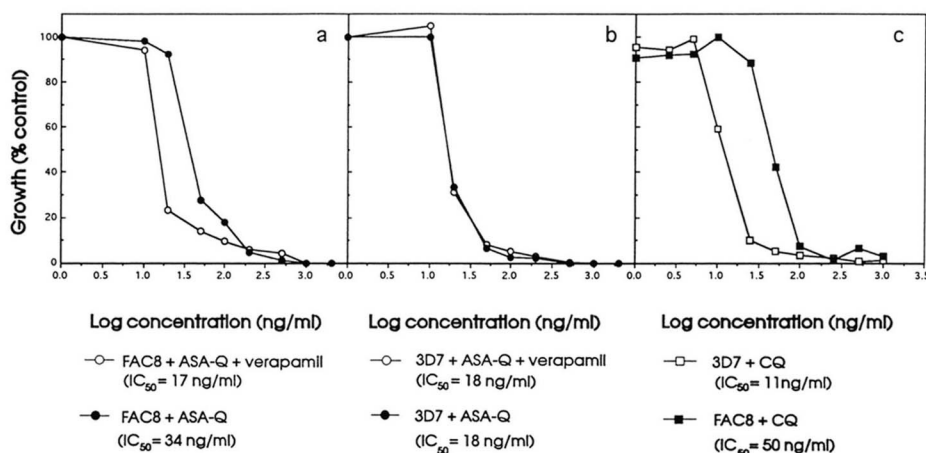


FIG. 2. **Effect of chloroquine and ASA-Q on the growth of malaria parasites *in vitro*.** Synchronized cultures of chloroquine-sensitive (3D7) and chloroquine-resistant (FAC8) *P. falciparum* were incubated with increasing concentrations of quinoline drugs, in the presence or absence of 1  $\mu\text{g}/\text{ml}$  verapamil. After 96 h of cultivation with daily replacement of drug-supplemented medium, the infected erythrocytes were harvested and growth was determined as a percentage of controls as described by Barnes *et al.* (1992). Each data point represents the mean of duplicate determinations.

quine-sensitive isolate 3D7 were isolated by flotation on a Percoll cushion. Mature stage-infected erythrocytes were incubated in the presence of iodo-ASA-Q (0.18 pmol of [monoiodo]ASA-Q, 0.04 pmol of [diiodo]ASA-Q, 0.46  $\mu\text{Ci}$ ), and photoactivated by exposure to light at 365 nm. SDS-PAGE and phosphorimage analysis revealed preferential labeling of proteins with apparent molecular masses of 42 and 33 kDa (Fig. 3, lane a). Neither the 42- nor the 33-kDa protein represents a major Coomassie Blue-staining protein (Fig. 3, lane d). A number of other proteins were weakly labeled by iodo-ASA-Q (Fig. 3); however, these proteins had molecular weights corresponding to major Coomassie Blue-staining proteins, and the level of labeling of these proteins was not decreased by the addition of excess chloroquine, indicating that they are labeled nonspecifically. The labeling of the 42- and 33-kDa proteins was only partly reduced by the inclusion of 1 mM  $\beta$ -mercaptoethanol in the incubation medium, while the nonspecific labeling of other proteins was largely abolished (data not shown). No labeling of proteins was observed in the absence of UV illumination. Labeling of both the 42- and 33-kDa proteins by iodo-ASA-Q was successively reduced by preincubation with increasing levels of unlabeled chloroquine (Fig. 3, lanes b and c). This suggests that chloroquine is competing with iodo-ASA-Q for the binding site(s) on these two proteins.

Fig. 4 shows a photoaffinity labeling experiment using uninfected erythrocytes. We observed no preferential labeling of proteins by iodo-ASA-Q in non-parasitized erythrocytes suggesting that both the 42- and 33-kDa proteins are of parasite origin.

Photoaffinity labeling studies were also performed using two

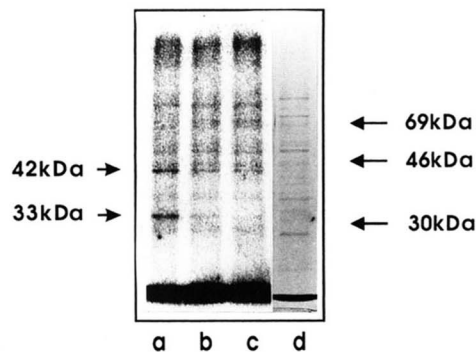


FIG. 3. **Photoaffinity labeling of chloroquine-interacting proteins in malaria-infected erythrocytes.** Erythrocytes infected with mature stages of a chloroquine-sensitive strain of *P. falciparum* (3D7) were incubated with iodo-ASA-Q (0.46  $\mu\text{Ci}$ , 4.4 nM) and exposed to UV illumination either alone (a) or in the presence of 4  $\mu\text{M}$  chloroquine (b) or 8  $\mu\text{M}$  chloroquine (c). Samples were subjected to SDS-PAGE (10% acrylamide) and visualized by phosphorimage analysis (Molecular Dynamics). Lane d is the Coomassie Blue-staining profile of lane a.

chloroquine-resistant strains of malaria parasites (Fig. 5). FAC8 has an  $\text{IC}_{50}$  value of 50 ng/ml for chloroquine (Fig. 2). K1mef is a parasite line derived from a cloned chloroquine-resistant isolate K1, which has been selected *in vitro* for resistance to mefloquine. K1mef has  $\text{IC}_{50}$  values of 240 ng/ml for chloroquine and 16 ng/ml for mefloquine.<sup>2</sup> The levels of photoaffinity labeling of the 42- and 33-kDa proteins (Fig. 5, a and

<sup>2</sup> A. F. Cowman, J. Thompson, and D. Galatis, (1994) *Proc. Natl. Acad. Sci. U. S. A.*, in press.

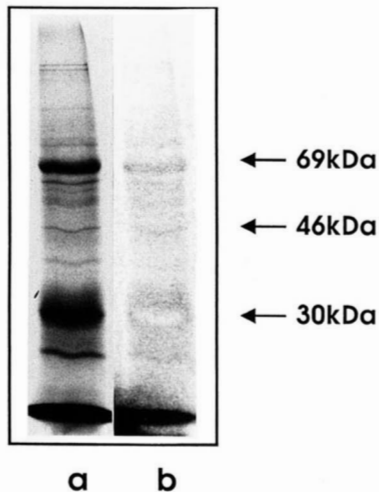


FIG. 4. Photoaffinity labeling of chloroquine-interacting proteins in uninfected erythrocytes. Human erythrocytes were washed to remove serum components, then incubated with iodo-ASA-Q (0.46  $\mu\text{Ci}$ , 4.4 nM) and exposed to UV illumination (b). The sample was subjected to SDS-PAGE (10% acrylamide) and visualized by phosphorimager analysis. Lane a is the Coomassie Blue-staining profile of lane b.

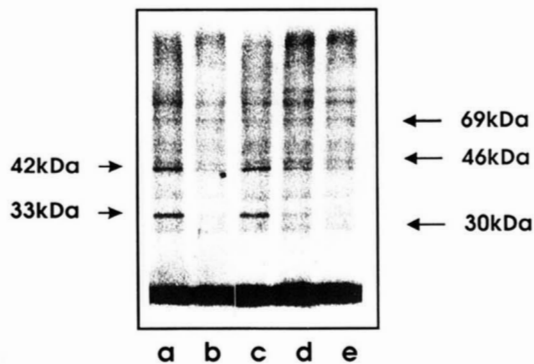


FIG. 5. Phosphorimage analysis of photoaffinity labeling of chloroquine-interacting proteins in erythrocytes infected with mature stages of chloroquine-resistant strains of *P. falciparum*. Erythrocytes parasitized with FAC8 (a and b) and K1mef (c-e) were photolabeled with 4.4 nM iodo-ASA-Q either alone (a and c) or in the presence of 4  $\mu\text{M}$  chloroquine (b and d) or 8  $\mu\text{M}$  chloroquine (e). Samples were subjected to SDS-PAGE (10% acrylamide).

c), and the patterns of competition (Fig. 5, b, d, and e) were similar to labeling patterns observed for the chloroquine-sensitive parasites (Fig. 3).

The ability of other drugs to competitively inhibit the photoaffinity labeling of proteins by iodo-ASA-Q was examined (Fig. 6). The specific labeling of parasite proteins in 3D7-infected erythrocytes was reduced by addition of excess unlabeled chloroquine or amodiaquine, and to a lesser extent, by quinine (Fig. 6, lanes b, d, and c). Densitometric analysis of competition data from three separate experiments suggests that, at the levels of competing drugs employed, chloroquine, amodiaquine, and quinine inhibit photoaffinity labeling of the 33-kDa protein by  $85 \pm 11$ ,  $71 \pm 2$ , and  $37 \pm 12\%$ , respectively. The three drugs inhibited labeling of the 42-kDa protein by  $75 \pm 7$ ,  $68 \pm 8$ , and  $33 \pm 5\%$ . Labeling of proteins was not inhibited by verapamil, a compound which modulates chloroquine resistance, nor by the structurally unrelated antibiotic antimalarial, doxycyclin (Fig. 6, lanes e and f). In the presence of excess mefloquine, labeling of both the 42- and 33-kDa proteins was substantially enhanced (Fig. 6, lane g), along with an increase in the level of nonspecific labeling of the major Coomassie Blue-staining proteins.

Fig. 7A presents data from an experiment in which erythro-

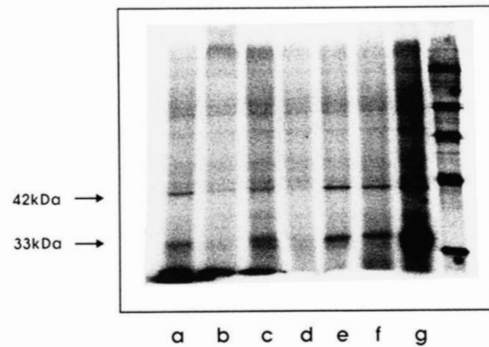


FIG. 6. Competitive inhibition of the photoaffinity labeling of malaria proteins by iodo-ASA-Q. Erythrocytes parasitized with the chloroquine-sensitive (3D7) strain were photolabeled with iodo-ASA-Q (0.46  $\mu\text{Ci}$ , 4.4 nM) either alone (a) or in the presence of 4.0  $\mu\text{M}$  chloroquine (b), or 4.8  $\mu\text{M}$  quinine (c), 4.8  $\mu\text{M}$  amodiaquine (d), 4.8  $\mu\text{M}$  verapamil (e), 4.8  $\mu\text{M}$  doxycyclin (f), or 4.8  $\mu\text{M}$  mefloquine (g). Samples were subjected to SDS-PAGE (10% acrylamide) and visualized using a Molecular Dynamics PhosphorImager. The last lane contains  $^{14}\text{C}$ -methylated protein markers; apparent molecular masses, from bottom to top, are 30, 46, 69, 97.4, and 200 kDa.

cytes, infected with mature stage parasites, were incubated with highly purified samples of the two different iodinated derivatives of ASA-Q, either [diiodo]ASA-Q (0.07 pmol, 0.23  $\mu\text{Ci}$ ) or [monoiodo]ASA-Q (0.13 pmol, 0.23  $\mu\text{Ci}$ ). [Diiodo]ASA-Q was found to preferentially label the 33-kDa protein (Fig. 7A, lanes a and b), while [monoiodo]ASA-Q preferentially labeled the 42-kDa protein (Fig. 7A, lanes c and d). As was found for the mixture of the mono- and di-iodinated species, the labeling of both the 33- and 42-kDa proteins, by the individual iodinated ASA-Q derivatives, was competitively inhibited by the addition of excess unlabeled chloroquine (Fig. 7, B and C and data not shown). By contrast, salicylamide, a weak base compound which is structurally related to the photoreactive moiety of ASA-Q did not inhibit labeling of either the 33- or 42-kDa proteins (Fig. 7A, lanes b and d). In the experiment presented in Fig. 7A, radiolabel was also incorporated into a number of other proteins, including polypeptides with approximate molecular masses of 90 and 12 kDa; however, this labeling was not inhibited by the addition of excess unlabeled chloroquine (data not shown), suggesting that it is nonspecific in nature.

Fig. 7 (B and C) shows the effect of solubilization of malaria-infected erythrocytes in a buffer containing 0.5% Triton X-100 (see "Materials and Methods") prior to photoactivation in the presence of [monoiodo]ASA-Q (0.26 pmol, 0.46  $\mu\text{Ci}$ ) or [diiodo]ASA-Q (0.08 pmol, 0.28  $\mu\text{Ci}$ ). Addition of excess unlabeled chloroquine specifically inhibited the labeling of the 42- and 33-kDa proteins (Fig. 7B, lanes b and c; Fig. 7C, lane b). If the parasitized erythrocytes were extracted with 0.5% Triton X-100 after photoaffinity labeling, both the 33- and the 42-kDa proteins were found to partition into the Triton X-100-soluble phase (data not shown). Fig. 8 shows densitometric analysis of the photoaffinity labeling of the Triton X-100-solubilized samples in the presence of increasing levels of [monoiodo]ASA-Q (Fig. 8a) and [diiodo]ASA-Q (Fig. 8b). Labeling of both the 42- and 33-kDa proteins exhibits saturation kinetics suggesting a limited number of binding sites for the chloroquine analogs.

#### DISCUSSION

A photoreactive analog of chloroquine ASA-Q has been synthesized as a photoaffinity probe for chloroquine-interacting proteins in malaria-infected erythrocytes. In order to establish ASA-Q as a suitable photoaffinity ligand for chloroquine-binding proteins, we examined its antimalarial activity. Indeed, ASA-Q turns out to be an effective antimalarial and was shown to possess more than 50% of the inhibitory activity of chloro-

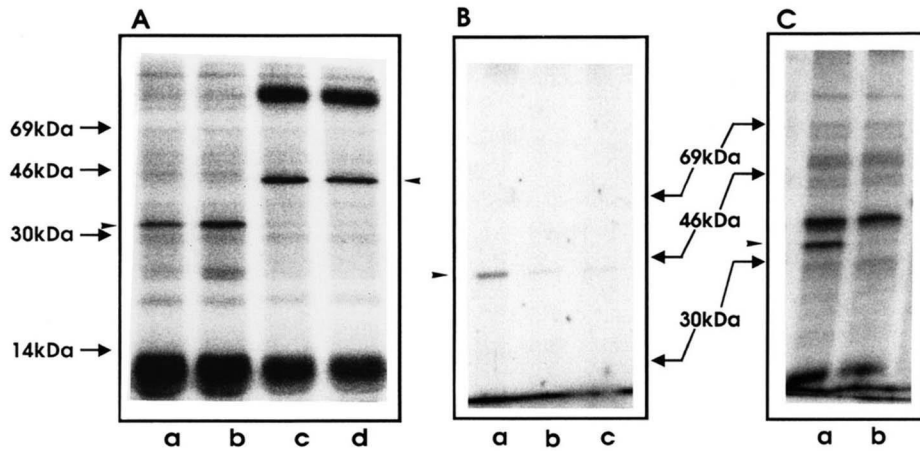


FIG. 7. Phosphorimage analysis of photoaffinity labeling of chloroquine-interacting proteins with separated mono- and di-iodinated derivatives of ASA-Q. Panel A, intact parasitized erythrocytes were incubated with either 1.3 nM [diiodo]ASA-Q (lanes a and b) or 2.6 nM [monoiodo]ASA-Q (lanes c and d) either in the presence (lanes b and d) or absence (lanes a and c) of salicylamide (100  $\mu$ M). Samples were subjected to SDS-PAGE (14% acrylamide). The 42- and 33-kDa proteins are indicated by arrowheads. Panel B, parasitized erythrocytes were solubilized in a buffer containing 0.5% Triton X-100 and 1 mM  $\beta$ -mercaptoethanol before labeling with 5.2 nM [monoiodo]ASA-Q either alone (lane a) or in the presence of 4  $\mu$ M chloroquine (lane b) or 8  $\mu$ M chloroquine (lane c). Samples were subjected to SDS-PAGE (10% acrylamide). The 42-kDa protein is indicated by an arrowhead. Panel C, parasitized erythrocytes were solubilized in 0.5% Triton X-100 prior to labeling with 1.6 nM [diiodo]ASA-Q either alone (lane a) or in the presence of 8  $\mu$ M chloroquine. Samples were subjected to SDS-PAGE (8–16% acrylamide). The 33-kDa protein is arrowed.

quine against a chloroquine-sensitive *P. falciparum* isolate 3D7. Although there is a quantitative difference in the antimalarial activities of chloroquine and ASA-Q, the data suggest that they act via a similar mechanism of action. ASA-Q therefore represents a suitable probe for photoaffinity labeling of proteins involved in chloroquine action. Furthermore, the potency of ASA-Q as an antimalarial suggests that it has a relatively high equilibrium association constant for binding to its target.

To determine whether the phenotype which confers chloroquine resistance is also associated with resistance to ASA-Q, the ability of ASA-Q to inhibit the growth of a chloroquine-resistant clone, FAC8, was examined. FAC8 was shown to be 2-fold more resistant to ASA-Q than the chloroquine-sensitive isolate 3D7. Moreover, the resistance of FAC8 to ASA-Q was modulated by verapamil, as has previously been demonstrated for chloroquine resistance (Martin *et al.*, 1987; Krogstad *et al.*, 1987; Barnes *et al.*, 1992). These data suggest that ASA-Q may be a suitable compound for probing resistance mechanisms, although it should be noted that the decrease in antimalarial activity against the resistant strain is somewhat less pronounced for ASA-Q than for chloroquine.

ASA-Q was readily radioiodinated with  $\text{Na}^{125}\text{I}$  and chloramine T. For the photolabeling experiments, we have prepared iodo-ASA-Q in a carrier-free state and used it at a concentration below the level at which it inhibits parasite growth (*i.e.* approximately 10-fold lower than the  $\text{IC}_{50}$  for sensitive parasites). Exposure of iodo-ASA-Q to UV light leads to photodecomposition of the aromatic azide producing highly reactive, short-lived, nitrene intermediates (Bayley and Knowles, 1977). The short half-life of the activated intermediates allows specific covalent labeling of proteins in malaria-infected erythrocytes that recognize the chloroquine structure of the analog. Indeed, photolabeling of malaria-infected erythrocytes with the radiolabeled probe identified two chloroquine-binding proteins with apparent molecular masses, 42 and 33 kDa. The labeling was absolutely dependent on UV irradiation implying that photolabeling occurs strictly through a nitrene intermediate (or its rearrangement products). Photoaffinity labeling of the two low abundance parasite proteins appears to be specific as it was competitively inhibited by chloroquine and two other quinoline antimalarials, amodiaquine and quinine, but not by structur-

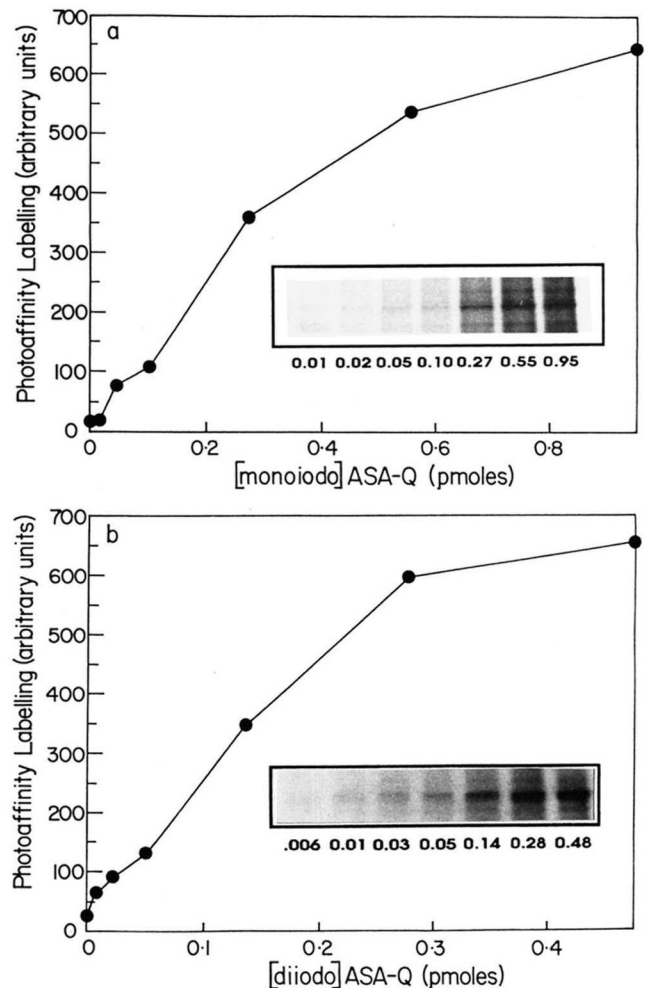


FIG. 8. Photoaffinity labeling of parasite proteins in the presence of increasing amounts of radiolabeled chloroquine analogs. Parasitized erythrocytes were solubilized in a buffer containing 0.5% Triton X-100 before labeling with either [monoiodo]ASA-Q (panel a) or [diiodo]ASA-Q (panel b). Samples were subjected to SDS-PAGE and incorporation of radiolabel into the 42- and 33-kDa proteins (see insets) was quantitated using densitometric analysis of the phosphorimage data.

ally unrelated drugs, nor by salicylamide, a weak base which contains structural elements of ASA-Q.

It has previously been suggested that chloroquine, amodiaquine, and quinine exert their antimalarial activities via a similar mechanism (Chou *et al.*, 1980). These antimalarials are structurally related and are all accumulated by malaria-infected erythrocytes with a similar efficiency (Chou *et al.*, 1980). These three quinoline antimalarials showed competitive inhibition of the photoaffinity labeling of the 42- and 33-kDa proteins, by iodo-ASA-Q, with the following relative efficiencies: chloroquine > amodiaquine >> quinine. This effect parallels the antimalarial activities of these drugs which supports the idea that the proteins identified in these studies are the macromolecular "targets" of quinoline drug action.

Surprisingly, a high molar excess of mefloquine appeared to enhance the level of photoaffinity labeling. In particular, the level of labeling of the 33-kDa protein was markedly increased. This increase in labeling may result from partial membrane disruption at the high levels (4.8  $\mu\text{M}$ ) of mefloquine used in this study. Mefloquine is a relatively hydrophobic drug and acts as an effective antimalarial in the concentration range 1–10 nM. At the drug levels used in the competition experiments, it may promote nonspecific interactions of iodo-ASA-Q with binding proteins. Alternatively, the data may reflect a physiologically important synergistic effect between chloroquine and mefloquine. Further studies are required to characterize this interaction in more detail.

Photoaffinity labeling experiments using highly purified preparations of the individual mono- and di-iodinated ASA-Q species reveal preferential labeling of the 42-kDa protein by [monoiodo]ASA-Q and preferential labeling of the 33-kDa protein by [diiodo]ASA-Q. The differential labeling of the two proteins by the two iodinated species may result from steric effects due to the fact that the [diiodo]ASA-Q is significantly larger and more polar than the monoiodinated derivative and may thus not gain access to the same labeling site. Whatever the reason for the differential labeling, our observations that chloroquine competes efficiently for the labeling sites on both proteins, and that salicylamide does not inhibit labeling, indicate that both the 42- and 33-kDa polypeptides are chloroquine-binding proteins.

Chloroquine accumulates to high levels in the digestive vacuole and, as the ASA-Q analog has similar pharmacological properties, and is also a diprotic weak base, it is anticipated that it is also concentrated in this compartment. The 42- and 33-kDa proteins may, thus, be present within the digestive vacuole where the target of chloroquine is presumed to be located. A number of proteases that play a role in hemoglobin digestion, and which are inhibited by chloroquine, have been identified in various *Plasmodium* species (Charet *et al.*, 1980; Gyang *et al.*, 1982; Sherman and Tanigoshi, 1983; Vander Jagt *et al.*, 1986, 1992; Goldberg *et al.*, 1991). Such proteases may correspond to the proteins labeled by iodo-ASA-Q. Alternatively, it is possible that either of the proteins could correspond to the putative heme polymerase that has recently been described by Slater and Cerami (1992) or the permease postulated by Warhurst (1986). It is also possible that the proteins identified by iodo-ASA-Q are novel.

A *P. falciparum* homolog (Pgh1) of the mammalian P-glycoprotein has been suggested to be involved in the mechanism of chloroquine resistance in some malaria isolates (Foote *et al.*, 1989, 1990; Barnes *et al.*, 1992). It has also been shown that heterologous expression of Pgh1 in Chinese hamster ovary cells confers increased chloroquine sensitivity, suggesting that this protein is involved in the concentration of chloroquine into the

digestive vacuole of *P. falciparum*.<sup>3</sup> Transcripts of a second P-glycoprotein homolog (Pgh2) have also been shown to be overexpressed in chloroquine-resistant parasites (Ekong *et al.*, 1993). In this study, we have examined two different strains of chloroquine-resistant *P. falciparum* both of which overexpress the 162-kDa Pgh1 protein (Cowman *et al.*, 1991).<sup>2</sup> Under the conditions of our experiments, we have not labeled any proteins the same size as Pgh1 or Pgh2. These data suggest that, while Pgh1 may play a role in the accumulation of chloroquine into the food vacuole, it does not seem to bind ASA-Q directly, or at least not with high affinity. Pgh1 may, however, influence the accumulation of chloroquine by an indirect mechanism such as regulation of the pH of this organelle (Barnes *et al.*, 1992).<sup>3</sup>

It is noteworthy that, under the conditions of our experiments, similar levels of labeling of the 42- and 33-kDa proteins were observed in both chloroquine-sensitive and chloroquine-resistant strains and that verapamil does not compete for labeling of either of these malarial proteins. The simplest interpretation of these findings is that neither the 33- nor the 42-kDa protein is involved in chloroquine resistance. It is possible that the chloroquine analog used in this study is not recognized by the protein(s) responsible for chloroquine resistance and further experiments, using alternative photoreactive chloroquine analogs are needed to clarify this situation; such experiments are currently in progress.

In conclusion, two chloroquine-binding proteins have been identified which may be involved in the mechanism of action of this important antimalarial drug. Unambiguous assignment of the roles of these proteins in the parasitic process requires purification and characterization of the 42- and 33-kDa polypeptides. We are currently undertaking the purification of these proteins in the anticipation that their precise characterization may assist in the development of novel antimalarial strategies.

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