Carrier-Linked Primaquine in the Chemotherapy of Malaria[1]

Jan Hofsteenge,*[†,‡] Anne Capuano,[†] Rita Altszuler,[§] and Stanford Moore[†,⊥]

The Rockefeller University, New York, New York 10021, and New York University, New York, New York 10010.
Received November 12, 1985

The antimalarial effect of intravenously administered primaquine (PQ) can be improved and its toxicity diminished by linking it to a macromolecular carrier protein. A thiol-containing primaquine derivative 8-[[4-(2-amino-3-mercaptopropionamido)-1-methylbutyl]amino]-6-methoxyquinoline was synthesized. This compound could readily be linked via a disulfide bond to a carrier protein containing (pyridyldithio)propionate groups. The derivative was coupled to serum albumin as well as to serum albumin that contained covalently linked lactose residues. The protein–drug conjugates were tested for their antimalarial activity in mice inoculated with *Plasmodium berghei*. The causal prophylactic activity of the conjugate with the lactosaminated serum albumin was 2 times higher than that of the free drug; the mean causal prophylactic doses (CPD_{50}) were 6 and 13 mg of primaquine base/kg, respectively. Moreover, its acute lethal toxicity had decreased at least 6.5-fold (mean lethal dose (LD_{50}) >85 mg of primaquine base/kg). The therapeutic index of this conjugate was at least 12 times higher than that of the free drug. This allowed the administration of a dose that cured 100% of the animals (17.5 mg of primaquine base/kg), in a single injection. With unmodified serum albumin the conjugate showed an increased therapeutic efficacy (the CPD_{50} was approximately 10 mg of primaquine base/kg) and a strongly reduced lethal toxicity.

Primaquine [8-[(4-amino-1-methylbutyl)amino]-6-methoxyquinoline] is widely used against the liver stages of the malaria parasite, in particular against the persistent stages of *Plasmodium vivax* and *Plasmodium ovale*.[2] However, its host toxicity poses a serious problem. Its hemolytic effect is especially a threat for individuals with a glucose-6-P-dehydrogenase deficiency. Efforts have been made to synthesize less toxic 8-aminoquinolines.[3]

An alternative approach to increase the therapeutic efficacy and/or reduce the toxicity is to target the drug more specifically to those cells that contain the parasite.[4] Encapsulating primaquine in liposomes reduced its acute lethal toxicity 3.6-fold but left the efficacy unchanged.[5] Linking the drug to a small peptide both reduced its toxicity and enhanced its therapeutic effect.[6]

A more specific targeting could perhaps be achieved by linking the drug to a macromolecular carrier protein. The linkage between the drug and the carrier protein should be relatively stable in the bloodstream but should readily be cleaved inside the target cell. Since free thiol in the blood is relatively low, whereas it is relatively high intracellularly, a disulfide bond should, in principle, provide a link that fulfills these requirements. Scheme I illustrates the reaction by which we proposed to link thiolated drug to a carrier protein. The sulfhydryl–disulfide interchange reaction proceeds efficiently under mild conditions. Moreover, the chemistry of the introduction of reversibly protected thiol groups into proteins has been well established.[7-10] We have linked primaquine to bovine serum albumin that contained covalently linked lactose residues (lac–BSA) and to unmodified bovine serum albumin (BSA). The former conjugate was expected to show improved uptake in the liver via the asialoglycoprotein receptor system. This receptor is present on the hepatocytes and removes glycoproteins with a terminal galactose from the bloodstream.[11,12] Inhibitors of DNA synthesis targeted by this method reduce DNA synthesis of *Ectromelia* virus in hepatocytes by about 50%. The required dose was 10 times lower than that of the free drug.[13]

We describe the synthesis of a thiol-containing primaquine derivative, its coupling to the carrier proteins, and the in vivo testing of the antimalarial activity and acute lethal toxicity of the protein–drug conjugates in a rodent model.[14] A very similar approach has been reported by Trouet et al.[15]

Scheme I

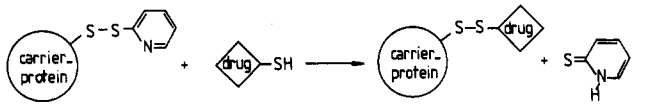
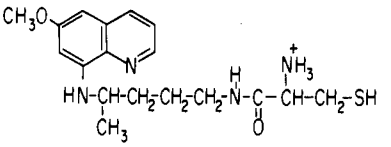


Chart I



Results

Protein–Drug Conjugate.[16] The coupling of cystein-

(1) A preliminary report of this work has been published in abstract form: Hofsteenge, J.; Capuano, A.; Altszuler, R.; Moore, S. *J. Cell. Biochem.* **1983**, *6*, 18.
(2) Thompson, P. E.; Werbel, L. In *Medicinal Chemistry*; Academic: New York, 1972; Vol. 12.
(3) Sweeney, T. R. In *Antimalarial Drugs II*; Peters, W., Richards, W. H. G., Eds.; Springer-Verlag: Heidelberg, 1984.
(4) Trouet, A. *Eur. J. Cancer* **1978**, *14*, 105–111.
(5) Pirson, P.; Steiger, R. F.; Trouet, A.; Gillet, J.; Herman, F. *Ann. Trop. Med. Parasitol.* **1980**, *74*, 383–391.
(6) Trouet, A.; Pirson, P.; Steiger, R.; Masquelier, M.; Baurain, R.; Gillet, J. *Bull. WHO* **1981**, *59*, 449–458.
(7) Carlsson, J.; Driven, H.; Axen, R. *Biochem. J.* **1978**, *173*, 723–737.
(8) King, T. P.; Li, Y.; Kochoumian, L. *Biochemistry* **1978**, *17*, 1499–1506.
(9) Jue, R.; Lambert, J. M.; Pierce, L. R.; Traut, R. R. *Biochemistry* **1978**, *17*, 5399–5405.
(10) White, F. H. *Methods Enzymol.* **1972**, *25B*, 541–546.
(11) Ashwell, G.; Morell, A. G. *Adv. Enzymol.* **1974**, *41*, 99–128.
(12) Ashwell, G.; Harford, J. *Ann. Rev. Biochem.* **1982**, *51*, 531–554.
(13) Fiume, L.; Busi, C.; Mattioli, A.; Balboni, P. G.; Barbanti-Brodano, G. *FEBS Lett.* **1981**, *129*, 261–264.
(14) Fink, E. *Bull. WHO* **1974**, *50*, 213–222.
(15) Baurain, R.; Deprez-De Campeneere, D.; Masquelier, M.; Pirson, P. In *Targeting of Drugs*; Gregoriades, G., Senior, J., Trouet, A., Eds.; Plenum: New York, 1982; pp 19–30.
(16) In addition to the primaquine derivative described below, two other thiol-containing derivatives were synthesized (i) 8-[[4-(3-mercaptopropionamidino)-1-methylbutyl]amino]-6-methoxyquinoline and (ii) 8-[[4-(3-mercaptopropionamido)-1-methylbutyl]amino]-6-methoxyquinoline. The first compound gave rise to sparsely soluble protein–drug conjugates, whereas the conjugate of the second compound was inactive at 4.4 mg of base/kg. For these reasons, further work on these derivatives was abandoned.

[†] The Rockefeller University.
[‡] Present address: Friedrich Miescher Institut, CH-4002 Basel, Switzerland.
[§] New York University.
[⊥] Deceased August 1982.

0022-2623/86/1829-1765$01.50/0

ylprimaquine (Chart I) to carrier protein modified with dithiopyridyl groups proved to be very efficient. The use of a thiolated drug equimolar to the amount of dithiopyridyl groups resulted in the coupling of 12.5 mol of drug/mol of conjugate (49 μg of primaquine base/mg of conjugate). The yield after gel filtration on Sephadex G-25, based on the amount of protein, was 85%.

Serum albumin is known to bind many drugs in a noncovalent fashion. To prove that cysteinylprimaquine was covalently linked to the protein and not simply adsorbed to it, the following experiments were performed on an analytical scale: (1) Thiolated drug was coupled to lactosaminated BSA-containing dithiopyridyl groups (lac-BSA-SP) (see methods). (2) lac-BSA-SP was incubated with primaquine (no thiol group). (3) lac-BSA (without dithiopyridyl groups) was incubated with thiolated primaquine. The mixtures were gel filtered on a column of Sephadex G-25 equilibrated with phosphate buffered saline (PBS). The protein peak of the real coupling (Figure 1A, supplementary material) contained primaquine as was evident from the absorbance at 353 nm. Moreover, a peak corresponding to the pyridine-2-thiol was observed (1.1 mol of pyridine-2-thiol/mol of primaquine). Very little drug, however (0.4–0.5 mol/mol), was found to be associated with the protein in the case of the controls (Figure 1B,C, supplementary material), and no peak at the position of pyridine-2-thiol was found. No free thiol could be detected in the protein–drug conjugate. From these results we conclude that the vast majority of the drug (>96%) was covalently linked to the carrier protein by means of a disulfide bond. More than 80% of the coupled drug could be released by incubating the conjugate with dithiothreitol. Chromatography of the protein–drug conjugate on Sephadex G-200 did not reveal cross-linking of the protein as a result of the modification with *N*-succinimidyl-3-(2-pyridyldithio)propionate or the coupling of the drug. The relative amount of dimer found in the conjugate was the same as in the native BSA preparation, i.e. 25%.

Stability of the Protein–Drug Conjugate in Serum. The protein–drug conjugate was incubated in vitro in serum to study the stability of the linkage between the protein and the drug. Thiol–disulfide interchange could liberate cysteinylprimaquine from the conjugate and result in a variety of products. Therefore, it was desirable to develop a procedure for the isolation of pure protein–drug conjugate from an incubation mixture containing serum, so that the amount of primaquine left in the conjugate could be determined.

We found that both lac–BSA and lac–BSA–PQ, containing 16–18 lactose residues, bound strongly to a column of immobilized (aminophenyl)boronic acid (Glycogel B). No serum proteins were detectable in the region in which lac–BSA–PQ eluted. Moreover, all the lac–BSA–PQ bound to the column and the recovery, based on A_{280}, was about 82%. Conjugate (11.6 or 7.3 mol of primaquine/mol of conjugate) was incubated at 37 °C for 1, 2, and 4 h with serum (2.4 mg of conjugate/mL of serum) and subsequently isolated by chromatography on Glycogel B. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate showed that the isolated conjugate was of high purity (Figure 2, supplementary material). Moreover, the amino acid composition of the isolated conjugate was in good agreement with that derived from the amino acid sequence of BSA[17] (data not shown) and was identical with a control that had not been incubated with serum, thus

(17) Dayhoff, M. O. *Atlas of Protein Sequences and Struture*; National Biomedical Research Center; Georgetown University Medical Center: Washington, DC, 1978.

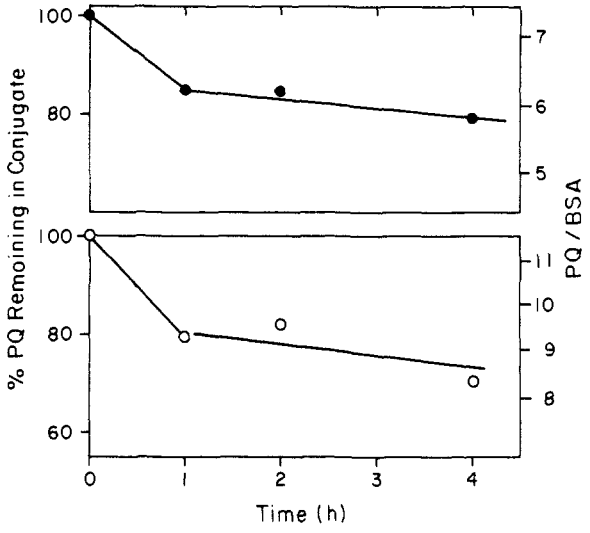


Figure 3. Stability of the protein–drug conjugate in serum. Conjugate that had been incubated with serum for various lengths of time was purified as shown in Figure 2 (supplementary material), and the ratio primaquine/conjugate was determined: (●) lac–BSA–$PQ_{7.3}$; (○) lac–BSA–$PQ_{11.6}$.

confirming the purity of the isolated conjugate. Only a very minor contaminating protein band was found. The mobility of this protein was slightly higher than that of lac–BSA and was identical with that of human serum albumin. The amount of the contaminant observed varied between different preparations of lac–BSA–PQ and was found to increase with increasing amounts of residual dithiopyridyl groups in the conjugate (results not shown). A protein band with the same mobility was found, when the conjugate was incubated with human serum albumin in PBS (35 mg of serum albumin/mL at 37 °C for 1 h). The appearance of this band was prevented by alkylating the serum with iodoacetate, prior to incubation with the conjugate. These results indicated that serum albumin could bind to the conjugate via thiol–disulfide interchange with residual dithiopyridyl groups in the conjugate. The conjugates used in the stability studies contained less than 0.7 mol of dithiopyridyl/mol.

The amount of primaquine remaining in the conjugate after incubation with serum decreased slightly with time (Figure 3). After a rapid initial loss of 15–20% of the drug, the release slowed down and 70–80% was still found in the conjugate after 4 h of incubation. The results, in terms of percentage loss, were very similar for the two preparations used. Conjugates incubated in PBS instead of serum did not lose any drug, and no A_{353} was detectable in lac–BSA, which had been incubated with serum and isolated by Glycogel chromatography.

Therapeutic Efficacy. To compare the therapeutic efficacy of free and protein-conjugated drug, a dose–response study was performed (Table I). The mean causal prophylactic dose (CPD_{50}) of primaquine, calculated from these data, was 13 mg of primaquine base/kg. lac–BSA–PQ was twice as effective as free primaquine: CPD_{50} = 6 mg of primaquine base/kg. In comparison, 7 mg/kg of free primaquine was ineffective (Table I). No curvative effect was found when lac–BSA without drug (350 mg of protein/kg, the amount of carrier for a dose of 17 mg of primaquine base/kg) was administered; all animals developed a parasitemia as quickly as the control.

If the increase in the therapeutic efficacy of lac–BSA–PQ were caused by an improved uptake into the liver via the asialoglycoprotein receptor system, one would predict that the protein–drug conjugate without lactose (BSA–PQ)

Table I. Curative Effect of Free and Protein-Conjugated Cysteinylprimaquine

compd	dose[a]	% cure	CPD_{50}
primaquine[b]	7	0	
	8	13	
	9.5	13	13
	11	20	
	14.5	69	
lac-BSA-PQ[b]	4	27	
	5	43	
	6.5	50	
	8	63	6
	10	63	
	10.5	88	
	17.5	100	
BSA-PQ	6	25	
	17.5	75	10
lac-BSA[c]	0	0	-
none	0	0	-

[a] Doses are milligrams of primaquine base/kilogram. [b] Pooled results of at least two experiments. [c] 350 mg of lac-BSA/kg was administered.

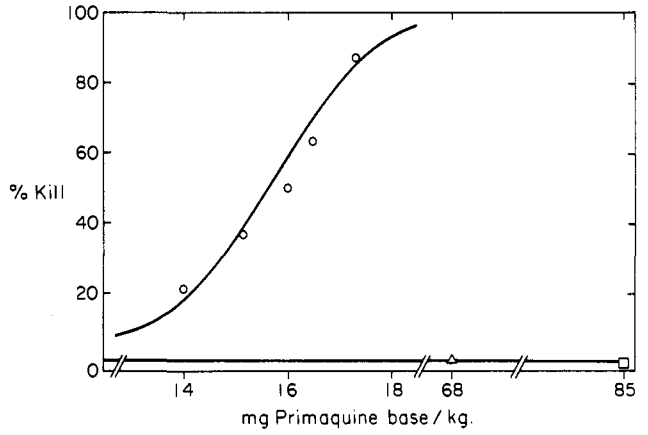


Figure 4. Acute lethal toxicity of primaquine and cysteinyl primaquine coupled to a carrier protein. Groups of eight mice were injected intravenously with either free drug (O), cysteinylprimaquine linked to lac-BSA (□), or cysteinylprimaquine linked to BSA (△). Doses are on a logarithmic scale. The curve drawn in the case of free primaquine is the theoretical curve, calculated from the probit transformed data.

would be less effective. The estimated CPD_{50} of BSA-PQ was about 10 mg of primaquine base/kg, or 1.7 times higher than that of lac-BSA-PQ. This suggests that the presence of lactose on the carrier protein played a minor role in increasing the therapeutic efficacy of the protein-drug conjugate. BSA-PQ was about as active as free primaquine, which means that the protein-conjugated drug can reach the parasite without making use of the asialoglycoprotein receptor.

Acute Lethal Toxicity. The acute lethal toxicity of intravenously administered primaquine and that of protein-drug conjugates was compared in noninfected animals (Figure 4). For primaquine, the LD_{10}, LD_{50}, and LD_{90} were calculated to be 13, 16, and 18 mg of primaquine base/kg, respectively. In contrast, no lethal effect was observed with lac-BSA-PQ. At the highest dose tested, 85 mg of primaquine base/kg, none of the treated animals (eight) died (Figure 4). Administration of higher doses was impractical because of the limited solubility of the conjugate (a solution of 70 mg of protein/mL had to be used to administer 85 mg of primaquine base/kg). Thus, 85 mg/kg was a minimum estimate for the LD_{50} (and LD_{10}) of this conjugate. A nonlethal toxicity was noticeable in the form of a loss of weight (about 10% compared to a control group). Two days after the injection the animals had regained their normal weight. The influence of the covalently linked lactose on the large reduction in toxicity was tested by injecting animals with a large dose of BSA-PQ (68 mg of primaquine base/kg). None of the eight animals injected with this conjugate died (Figure 4).

Table II. Summary of Therapeutic Efficacy and Acute Lethal Toxicity of Free and Protein-Linked Cysteinylprimaquine[a]

	CPD_{50}	CPD_{90}	LD_{10}	LD_{50}	LD_{50}/CPD_{50}
primaquine	13	20	13	16	1.2
lac-BSA-PQ	6	15	>85	>85	>14.2
BSA-PQ	10	ND	>68	>68	>6.8

[a] Doses in milligrams of primaquine base/kilogram.

The therapeutic index (LD_{50}/CPD_{50}) of lac-BSA-PQ calculated from these data was at least 12 times higher than that of the free drug (Table II). The ratio LD_{10}/CPD_{90} shows an increase of at least 7 times.

Discussion

In this study we have used thiol-disulfide interchange to couple primaquine to BSA and lac-BSA through a disulfide bond, with the aim of obtaining a more effective and less toxic protein-drug conjugate. The results presented demonstrated a number of the advantages of the use of thiol-disulfide interchange to link a drug to a carrier. Reacting lac-BSA-SP with 1 equiv of the thiolated drug resulted in a high amount of coupled drug. (generally 85%). Despite the high number of cysteinylprimaquine molecules linked, no cross-linking of the protein-drug conjugate was found. This was an advantage over methods that use symmetrical bifunctional reagents, e.g. glutaraldehyde,[18] or carbodiimides[19] for the coupling of drugs to proteins. Although the disulfide bond was not absolutely stable in serum in vitro, the majority of disulfide-linked primaquine (70–80%) was still found in the conjugate after 4 h of incubation.

The results obtained on the therapeutic efficacy of primaquine administered by the intravenous route (CPD_{50} = 13 mg of primaquine base/kg) were in agreement with the results of Trouet and collaborators (CPD_{50} = 13–14 mg of primaquine base/kg).[15] Primaquine linked to lac-BSA was about twice as effective (CPD_{50} = 6 mg of base/kg). The protein-drug conjugate without lactose residues was approximately 1.7 times less effective than lac-BSA-PQ, and somewhat more effective than free primaquine. This suggested that the presence of covalently linked lactose residues played no major role in increasing the therapeutic efficacy of the protein-drug conjugate.

The LD_{50} of free primaquine found in this study was lower than that found by Trouet et al.[15] (22 mg of primaquine base/kg). This could be due to the difference in the strains of mice used. Primaquine linked to a carrier protein was found to have a strongly reduced lethal toxicity. Since the toxicity of primaquine linked to both lac-BSA and BSA was reduced, one can conclude that there was no effect of the covalently linked lactose and that coupling to a macromolecular carrier was sufficient to reduce its acute lethal toxicity. Encapsulating primaquine in liposomes also diminished its acute lethal toxicity,[5] although not as much as linking it to a protein carrier, as shown in this paper.

The therapeutic index of lac-BSA-PQ had increased at least 12-fold compared with the free drug (Table II). This allowed the administration of a single curative dose: 17.5

(18) Hurwitz, E.; Levy, R.; Maron, R.; Wilchek, M.; Arnon, R.; Sela, M. *Cancer. Res.* **1975**, *35*, 1175–1181.
(19) Trouet, A.; Masquelier, M.; Baurain, R.; Deprez-De Campeneere, D. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 626–629.

mg of primaquine base/kg, in the form of lac-BSA-PQ, cured 100% of the animals. This dose was still 5 times lower than the minimum estimate of the LD_{10} for this conjugate.

Recently, Trouet et al.[15] also reported a 2-fold increase in therapeutic efficacy for their protein-drug conjugate (primaquine coupled to asialofetuin by means of the tetrapeptide alanylleucylalanylleucine). This conjugate also had a strongly reduced acute lethal toxicity (Pirson, P. personal communication).

Our results did not completely rule out the possibility that the observed increase in therapeutic index was simply due to the modification of primaquine with the cysteinyl moiety. However, experiments to test this possibility were impossible, because of the rapid oxidation of cysteinylprimaquine at pH 7.4 to the insoluble disulfide form (cystinylprimaquine). However, Trouet et al. have shown that linking primaquine to a single amino acid (leucine) was without effect on the therapeutic efficacy and toxicity. At least a dipeptide is required to obtain some improvement (2.5-fold) in therapeutic index.[6] Even the linkage to a tetrapeptide did not show the large increase in therapeutic index that we found for the protein-drug conjugate. Therefore, it seems likely that the large improvement in therapeutic index seen in our study is caused by the conjugation to the carrier protein.

Our studies did not provide a clue to the mechanism by which the linkage between primaquine and the drug was cleaved. Our rationale for using a disulfide bond was that the high intracellular free thiol concentration might liberate the drug either in its free thiol form or as a mixed disulfide. If the protein-drug conjugate is taken up into the cell intact, it is likely that it is degraded in the lysosomes. It has been suggested that cysteamine is the reductant for disulfides of proteins that are degraded inside the lysosomes.[20] Preliminary results showed that the disulfide bond between cysteinylprimaquine and lac-BSA could be reduced by incubation with cysteamine at pH 5.0. Alternatively, it is conceivable that primaquine is released from the conjugate by action of lysosomal proteases on the amide bond with cysteine.

Experimental Section

High-performance liquid chromatography was performed on a Beckman Model 334 liquid chromatograph. Spectra were recorded with an Aminco DW2 UV/vis spectrophotometer. Elemental analyses were performed by the microanalytical service department of the Rockefeller University. Analyses were within 0.2% of the theoretical values. The following materials and animals were obtained from the source listed: BSA (fraction V) was obtained from Miles. Primaquine diphosphate and sodium cyanoborohydride were from Aldrich. *N*-Succinimidyl-3-(2-pyridyldithio)propionate, triethylamine, and Glycogel B were purchased from Pierce. *N*-Boc-L-cystine *p*-nitrophenyl ester was a generous gift from Dr. S. A. Kahn, Rockefeller University. α-Lactose, dithiothreitol, and 5,5′-dithiobis(2-nitrobenzoic acid) were obtained from Sigma. Sep-Pak C_{18} cartridges and reversed-phase high-performance liquid chromatography columns were from Waters Associates. Sephadex G-25 was purchased from Pharmacia. Female A/J mice, 5–8 weeks old (about 20 g), were obtained from Jackson Laboratories, Bar Harbor, Me.

High-Performance Liquid Chromatography. A μBondapak C_{18} column, 0.4 × 30 cm or 0.8 × 30 cm for analytical and preparative purposes, respectively, was used. Primaquine or its derivative was eluted with a gradient of methanol (10–70% in 60 min) in 0.1% trifluoroacetic acid. The flow rate was 1.0 mL/min for the analytical and 3.0 mL/min for the preparative column. Compounds were detected by monitoring A_{280} of the eluate. For the determination of primary amines, the relative fluorescence was measured with on-line detection after reaction of the eluate with fluorescamine.[21,22]

Thiol Determination. The thiol content of the primaquine derivatives was determined by measuring the thiol concentration of a solution by the method of Ellman[23] using $\epsilon_{412} = 14\,200\ M^{-1}\ cm^{-1}$[24] and calculating the primaquine concentration from the absorbance at 353 nm.

Reductive Lactosamination. BSA was lactosaminated by the method of Gray,[25,26] essentially as described by Wilson.[27] The extent of the modification was determined from the difference in lysine content before and after the reaction.[28] Amino acid analysis was performed on a Dionex-500 analyzer, after acid hydrolysis in 6 N HCl, for 24 h at 110 °C. Generally, after 48 h of incubation at pH 7.8 and 37 °C, 16–18 mol of lactose/mol of BSA had been coupled.

Introduction of 3-(2-Pyridyldithio)propionate Groups into (Lactosaminated) Serum Albumin. Protein (lactosaminated) was reacted with *N*-succinimidyl-3-(2-pyridyldithio)propionate by the procedure of Carlsson et al.[7] Excess reagent was removed by dialysis against 0.1 M potassium phosphate, pH 7.0 at 4 °C. The protein concentration was determined by the method of Lowry et al.[29] and the 2-pyridyl disulfide content by reading the increase in A_{343} after the addition of 100 μL of 0.1 M dithiothreitol to 900 μL of protein solution. On the average, 14–15 mol of (pyridyldithio)propionate were incorporated per mole of lac-BSA.

Cystinylprimaquine. Cystinylprimaquine was prepared as follows: 272 mg of primaquine diphosphate (0.6 mmol) and 204 mg of *N*-Boc-cystine *p*-nitrophenyl ester (0.3 mmol) in 1 mL dimethylformamide (titrated to pH 8 with triethylamine) were reacted overnight at room temperature. The reaction mixture was diluted with 40 mL of water, and the precipitate was collected by centrifugation (8000*g*, 60 min). The precipitate was washed twice with water and lyophilized. The Boc group of the crude *N*-Boc-cystinylprimaquine was removed by incubation in trifluoroacetic acid/CH_2Cl_2 (1:1, v/v) for 30 min at room temperature.[30] Trifluoroacetic acid and CH_2Cl_2 were removed with a jet of N_2. Cystinylprimaquine was isolated in 15-mg portions by high-performance liquid chromatography on a semipreparative C_{18} column. The product was lyophilized, yielding the cystinylprimaquine as the trifluoroacetic acid salt. The amorphous yellow powder was stored at −20 °C. Yield: 39%. Anal ($C_{44}H_{54}N_8O_{12}S_2F_{12}$) C, H, N. Reinjection into a C_{18} reversed-phase column gave a single peak (Figure 5, supplementary material).

Cysteinylprimaquine. Shortly before the drug derivative was coupled to the protein, the disulfide was reduced: 30 mg of cystinylprimaquine was dissolved in 2 mL of water, under N_2, 50 mg of dithiothreitol was added (10-fold excess), and the pH was brought to 7.5 with triethylamine. After 60 min at room temperature the pH was lowered to 2.5 with trifluoroacetic acid. Excess reagents were removed by chromatography on a silica-based C_{18} column (a 2 × 7 cm glass column filled with the contents of 30 Sep-Pak C_{18} cartridges), equilibrated with 10% methanol in 0.1% trifluoroacetic acid. After the column was washed with this eluent to remove all dithiothreitol, the cysteinylprimaquine was eluted with 70% methanol in 0.1% trifluoroacetic acid. Methanol was removed by rotary evaporation, and the remaining solution was used immediately in the coupling procedure. The

(20) Kooistra, T.; Millard, P. C.; Lloyd, J. B. *Biochem. J.* **1982**, *204*, 471–477.
(21) Udenfriend, S.; Stein, S.; Boehlen, P.; Dairman, W.; Leimgruber, W.; Weigele, M. *Science (Washington, D.C.)* **1972**, *178*, 871–872.
(22) Boehlen, P.; Stein, S.; Stone, J.; Udenfriend, S. *Anal. Biochem.* **1975**, *67*, 438–445.
(23) Ellman, G. L. *Arch. Biochem. Biophys.* **1959**, *82*, 70–77.
(24) Riddles, P. W.; Blakeley, R. L.; Zerner, B. *Methods Enzymol.* **1983**, *91*, 49–60.
(25) Gray, G. R. *Arch. Biochem. Biophys.* **1974**, *163*, 426–428.
(26) Schwartz, B. A.; Gray, G. R. *Arch. Biochem. Biophys.* **1977**, *181*, 542–549.
(27) Wilson, G. *J. Biol. Chem.* **1978**, *253*, 2070–2072
(28) Wilson, G. *J. Gen. Physiol.* **1979**, *74*, 495–509.
(29) Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.* **1951**, *193*, 265–275.
(30) Gutte, B.; Merrifield, R. B. *J. Biol. Chem.* **1971**, *246*, 1922–1941.

thiol content was 0.96 mol of SH/mol. Vis: λ_{max} = 353 nm (pH 7.5). The product gave a reaction with fluorescamine, indicating the presence of a primary amino group. Reinjection into a C_{18} reversed-phase column gave a single peak (Figure 5, supplementary material).

lac-BSA-PQ and BSA-PQ. Cysteinylprimaquine was coupled to 100 mg of BSA, or lac-BSA, containing 14–15 mol of (pyridyldithio)propionate groups/mol of protein in 50 mL of 0.1 M sodium phosphate, pH 7.2, 1 mM EDTA (the buffer was purged with N_2). Aliquots of 3 mL of cysteinylprimaquine (21–22.5 μmol in 30 mL of dilute trifluoroacetic acid, purged with N_2) were added at 3-min intervals. The reaction was performed at room temperature, under N_2 and was carried on for 30 min after the last addition. The protein–drug conjugates were purified by gel filtration at 4 °C on a column of Sephadex G-25, equilibrated with PBS, purged with N_2. The protein peak was pooled, and the primaquine content was calculated from the A_{353}, using a molar extinction of 3300 M^{-1} cm^{-1}. The protein concentration was determined by the method of Lowry et al.[29] The values were corrected for the interference of primaquine (1 μg of primaquine base gives the color equivalent to 3.64 μg of BSA). The protein solutions were concentrated in an Amicon Diaflo apparatus using a UM10 filter. The protein–drug conjugates were stored as a lyophilized powder at −20 °C; shortly before use, the complex was dissolved in PBS, dialyzed, and centrifuged.

Measurement of the Stability of the Protein–Drug Conjugate in Vitro. Serum was prepared from blood of a healthy individual. Conjugate (11.6 mol, or 7.3 mol of primaquine base/mol of conjugate) was incubated at 37 °C with serum for various lengths of time (2.4 mg of conjugate/mL of serum). In a control experiment PBS was substituted for serum. To prevent any sulfhydryl–disulfide interchange during subsequent analysis, 600 μL of incubation mixture was alkylated by the addition of 75 μL of 2 M Tris-HCl, pH 8.6, and 75 μL of 1 M iodoacetic acid (neutralized with NaOH) and incubation at room temperature in the dark for 30 min. After addition of $MgCl_2$ to a final concentration of 50 mM, the alkylated incubation mixture was chromatographed on a column of Glycogel B [(aminophenyl)-boronic acid immobilized on Sepharose CL 6B; 1.5-mL bed volume in a Pasteur pipet]. The column was equilibrated with 0.25 M ammoniumacetate, pH 8.5; 50 mM $MgCl_2$, 250 mM NaCl, 2 M urea, and a modification of the procedure of Mallia et al.[31] was used. After the column was washed with 11 mL of equilibration buffer, bound material was eluted with a linear gradient of 0–0.2 M sorbitol in 100 mM Tris-HCl, pH 8.5, 50 mM EDTA, 250 mM NaCl, 2 M urea (10 mL in each gradient vessel). After 11 mL of the gradient had passed through, elution was continued with 2 M sorbitol in the same buffer. The fraction size was 0.53 mL. Purified conjugate, after dialysis against PBS and lyophilization, was analyzed for protein and primaquine as described above.

Therapeutic Studies. The procedures for growing the parasites and the isolation of sporozoites were as described by Vanderberg et al.[32] Groups of six to eight female A/J mice (about 20 g) were inoculated with 10^4 sporozoites via the tail vein. Drug, protein–drug conjugate, or protein alone, dissolved in 0.5 mL of PBS, was injected intravenously 2–3 h after inoculation. In all studies the parasitemia was assessed by examination of Giemsa-stained thin bloodsmears up to the 12th day after inoculation. In every experiment one group of animals received no drug treatment and acted as a control. Animals that did not show parasitemia by the 12th day were regarded as cured.[14] The mean causal prophylactic dose was determined from dose–response studies after probit transformation of the data.[33]

Acute Lethal Toxicity Studies. Groups of eight mice were injected intravenously with varying amounts of free primaquine or cysteinylprimaquine coupled to BSA or lac-BSA. Freshly prepared solutions in PBS were used. Controls received PBS only. The LD_{50} value was determined after probit transformation of the data.[33]

Acknowledgment. We thank Dr. P. Blackburn for the suggestion of using a disulfide to couple drugs to proteins, Dr. R. S. Nussenzweig, New York University, in whose laboratory the therapeutic studies were performed, Marilyn Maracic for excellent technical assistance, and Drs. E. Shaw, S. R. Stone, and E. J. Mackie for critically reading the paper. We are thankful to Dr. J. M. Manning for his continued interest. This work was supported in part by NIH Grant GM-25323.

Registry No. Cysteinylprimaquine, 102615-50-9; cystinylprimaquine, 102615-51-0; primaquine diphosphate, 63-45-6; *N*-Boc-cystine *p*-nitrophenyl ester, 51077-22-6; primaquine, 90-34-6.

Supplementary Material Available: Figures 1, 2, and 5 showing coupling of cysteinylprimaquine to lactosaminated BSA, chromatography of lac-BSA, and HPLC of the primaquine derivative, respectively (2 pages). Ordering information is given on any current masthead page.

(31) Mallia, A. K.; Hermanson, G. T.; Krohn, R. I.; Fujimoto, E. K.; Smith, P. K. *Anal. Lett.* 1981, *14*, 649–661.

(32) Vanderberg, J. P.; Nussenzweig, R. S.; Most, H. *J. Parasitol.* 1968, *54*, 1009–1016.

(33) Finney, D. J. *Probit Analysis*; Cambridge University Press: Cambridge, England, 1971.

Potential Antitumor Agents. 47. 3′-Methylamino Analogues of Amsacrine with in Vivo Solid Tumor Activity

Graham J. Atwell, Bruce C. Baguley, Graeme J. Finlay, Gordon W. Rewcastle, and William A. Denny*

Cancer Research Laboratory, University of Auckland School of Medicine, Private Bag, Auckland, New Zealand.
Received November 7, 1985

Replacement of the 3′-methoxy group of the clinical antileukemic agent amsacrine (1) with a 3′-methylamino group provides a compound (3) with a broader spectrum of action, including in vivo activity against experimental solid tumors. The synthesis, physicochemical properties, and biological activity of a series of acridine-substituted analogues of 3 are described. The compounds show higher levels of DNA binding, water solubility, and in vivo solid tumor activity (Lewis lung carcinoma) than their amsacrine counterparts. However, the structure–activity relationships for acridine substitution are different, with 3,5-disubstituted 3′-methylamino compounds showing the highest activity (compared to 4,5-disubstituted amsacrine analogues).

Considerable clinical experience with the DNA-binding agent amsacrine (*m*-AMSA; 1) has shown it to be an effective drug for the treatment of leukemias and lymphomas, and it is now being employed as a component of drug

0022-2623/86/1829-1769$01.50/0