A Prodrug Form of a *Plasmodium falciparum* Glutathione Reductase Inhibitor Conjugated with a 4-Anilinoquinoline

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Glutathione (GSH), which is known to guard *Plasmodium falciparum* from oxidative damage, may have an additional protective role by promoting heme catabolism. An elevation of GSH content in parasites leads to increased resistance to chloroquine (CQ), while GSH depletion in resistant *P. falciparum* strains is expected to restore the sensitivity to CQ. High intracellular GSH levels depend inter alia on the efficient reduction of GSSG by glutathione reductase (GR). On the basis of this hypothesis, we have developed a new strategy for overcoming glutathione-dependent 4-aminoquinoline resistance. To direct both a 4-aminoquinoline and a GR inhibitor to the parasite, double-drugs were designed and synthesized. Quinoline-based alcohols (with known antimalarial activity) were combined with a GR inhibitor via a metabolically labile ester bond to give double-headed prodrugs. The biochemically most active double-drug **7** of this series was then evaluated as a growth inhibitor against six *Plasmodium falciparum* strains that differed in their degree of resistance to CQ; the ED_{50} values for CQ ranged from 14 to 183 nM. While the inhibitory activity of the original 4-aminoquinoline-based alcohol followed that of CQ in these tests, the double-drug exhibited similar efficiency against all strains, the ED_{50} being as low as 28 nM. For the ester **7**, a dose-dependent decrease in glutathione content and GR activity and an increase in glutathione-*S*-transferase activity were determined in treated parasites. The drug was subsequently tested for its antimalarial action in vivo using murine malaria models infected with *P. berghei*. A 178% excess mean survival time was determined for the animals treated with 40 mg/kg **7** for 4 days. No cytotoxicity due to this compound was observed. Work is in progress to extend and validate the strategy outlined here.

Introduction

Tropical malaria, a major health problem in many southern countries, is caused by multiplication of the protozoan parasite *Plasmodium falciparum* in erythrocytes. More than 400 million disease cases with over 2 million deaths is the annual toll of *P. falciparum* infections. *Roll back malaria* programs[1,2] are hampered inter alia by the spreading resistance of the parasite to standard antimalarials drugs, in particular to chloroquine (CQ), which had been the affordable and effective antimalarial mainstay for 50 years. Global loss of CQ effectiveness has led to intense research on the mode(s) of action of this drug.[3] The final goal of these studies is, of course, the reversal of parasite CQ tolerance. For understanding the action of CQ, it is relevant that the actual disease malaria is caused by multiplication of the parasites in human red blood cells. During digestion of host cell hemoglobin by the parasite, large amounts of free heme are produced in the food vacuole. Most of this is polymerized to inert hemozoin, while the residual heme diffuses into the parasite's cytosol where it is detoxified by interaction with glutathione (GSH).[4] The association constant of the hemin−GSH adduct is 3300 M^{-1} at pH 7.4.[5] CQ is assumed to act both by blocking heme polymerization[6,7] and by perturbing heme conjugation with glutathione.[8,9] The CQ-induced heme accumulation in cytosol and membranes of the parasite causes or at least contributes to cell death.

The mechanisms of chloroquine resistance are still controversially discussed.[10−19] However, one way to suppress CQ tolerance would be a drug formula that contains CQ in combination with a CQ-sensitizing compound. In the search for possible CQ sensitizers, obvious candidates are agents that destabilize the pool of intraparasitic glutathione. This follows from the CQ action described above. Furthermore, as also observed in tumor cells, an elevation of GSH content in parasites leads to increased resistance to several drugs.[8] The tripeptide GSH has numerous functions including protection of cells from oxidative stress and from electrophilic toxic compounds including heme. Intracellular

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GSH levels depend on numerous factors but predominantly on the de novo synthesis from the three amino acids Glu, Cys, and Gly[20] and on the efficient reduction of glutathione disulfide (GSSG) by the flavoenzyme glutathione reductase (NADPH + H^+ + GSSG → $NADP^+$ + 2 GSH).[21−24] Consistently, GSH depletion by L-buthionine-(*S,R*)-sulfoximine (BSO), an inhibitor of GSH synthesis, in resistant *P. falciparum* strains restores the sensitivity to CQ and its 4-aminoquinoline analogues.[8]

We focused on GR inhibitors as CQ sensitizers because these compounds are promising antimalarial agents on their own merits. Different series of GR inhibitors including nitrosoureas,[25] quinones,[26−29] 3,7-diamino-2,8-dimethyl-5-phenylphenazinium chloride (safranin),[27] 6-hydroxy-3-oxo-3*H*-xanthene-9-propionic acid,[30] 10-arylisoalloxazines,[31,32] the dye methylene blue,[33] and ajoene[34] have been described. GSH depletion in *P. falciparum* trophozoite-infected red blood cells by GR inhibitors such as 1,3-bis(2-chloroethyl)-1-nitrosourea, 10-arylisoalloxazines, methylene blue, and ajoene confers a drastic antiplasmodial effect.[25,33−36] Noteworthy is that three base exchanges found in the GR cDNAs from the chloroquine-sensitive strain 3D7 and the chloroquine-resistant strain K1 resulted in a GR with a 5-fold catalytic efficiency to regenerate GSH.[19]

In the present paper, we describe the screening of a library of 1,4-naphthoquinones (NQ), which led to the identification and characterization of several NQ alcanoic acids as novel GR inhibitors. From these lead structures, a series of prodrug esters were designed and synthesized in order to improve the cell penetration properties of the otherwise negatively charged agents. Subsequently, double-headed prodrugs with superior pharmacokinetic properties were prepared. These prodrugs contain an NQ alcanoic acid conjugated with a quinoline-based structure via an enzyme-sensitive ester bond. The quinoline is expected to target the compound to the parasite where the labile bond is hydrolyzed so that the GR inhibitor and the aminoquinoline are released as antimalarial principles at the site(s) of action. The most potent double-drug **7** corresponds to a prodrug ester of the most active *Plasmodium falciparum* GR (PfGR) inhibitor conjugated with a 4-anilinoquinoline. This double-drug was successfully tested in vitro against both CQ-resistant and CQ-sensitive *P. falciparum* strains and in vivo against *P. berghei* in infected mice. To gain a better insight in its mechanism of action, GSH content and GR activity were determined in parasites exposed to this double-drug.

Results

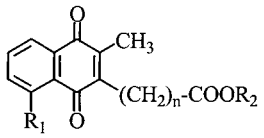
Identification of a Lead Structure by Screening a Library of Naphthoquinone Derivatives. Automated high-throughput GR inhibitor screening (HTS) was performed in microtiter plates by using a colorimetric assay based on reduction of glutathione disulfide in the presence of DTNB as a thiol-indicator reagent.[37] The library of 1400 naphthoquinone derivatives[38] was screened against hGR or PfGR at 25 μM test compound. For each NQ tested, the percentage of inhibition was calculated and compared to that of the GR inhibitor 10-(3′,5′-dichlorophenyl)-3-methylisoalloxazine (isoalloxazine **3b** in ref 36). The highest inhibition activities were found for the [2-(3-methyl)naphthoquinolyl]alcanoic acids of the menadione, plumbagin, and juglone series shown in Table 1 (see also Table 1 in Supporting Information). The most active compounds led to more than 80% inhibition of both hGR and *P. falciparum* GR. With respect to side chain length *n* of the inhibitors, the best results were obtained for $n = 4$ or 5 toward both GRs in the menadione series, for $n = 2$ toward hGR, and for $n = 2-4$ toward PfGR in the juglone series; in the plumbagin series the optimal value for *n* varied between 2 and 5 (see Table 1 in Supporting Information).

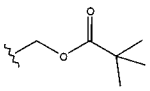
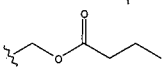
GR Inhibition Studies. To quantify the effects of the four most active GR inhibitors (**M_4**, **M_5**, **P_4**, and **P_5**), their IC_{50} values were measured in the standard GSSG reduction assay using 100 μM NADPH (Table 1). The optimum spacer length was found to be $n = 5$ toward both GR species. An IC_{50} value of 0.5 μM was determined in the presence of 100 μM GSSG for the most potent PfGR inhibitor 6-[2-(3-methyl)naphthoquinolyl]-hexanoic acid (**M_5**). The inhibition was fully reversible by dilution. In the presence of 40−300 μM of the substrate GSSG and 0−10 μM **M_5**, a competitive type of inhibition with a K_i value of 0.5 μM was deduced from a Cornish−Bowden plot (see Figure 1 in Supporting Information). This is consistent with the binding of **M_5** to the GSSG site of *P. falciparum* GR. The change in inhibition type observed at >1 mM GSSG may be explained by an additional binding site for **M_5**; this assumption is indirectly supported by crystallographic[39] and kinetic studies[40] on menadione as an inhibitor of human GR. Assuming competitive inhibition also for the other inhibitors, $K_{i,c}$ values were calculated for **M_4**, **M_5**, **P_4**, and **P_5** (Table 1). The data show that **M_5** is the most promising lead compound. Of all inhibitors, it has the lowest K_i value with the parasite enzyme and it inhibits the parasite enzyme more strongly than the human enzyme. The less effective inhibition of the human enzyme by **M_5** is not unwanted because human erythrocyte GR is also a potential target in antimalarial chemotherapy.[22] Although the bis(carboxylic acids) in the juglone series, for instance, **J_2**, discriminate between the human and parasite enzyme, they were not studied further (see Table 1 in Supporting Information).

Chemistry of M_5-Based Esters. Briefly, compounds **1** and **4**−**9** were prepared from the 6-[2-(3-methyl)-naphthoquinolyl]hexanoic acid **M_5** and the respective alcohol by esterification in the presence of dicyclohexylcarbodiimide (DCC) and 4-(dimethylamino)pyridine (DMAP). Compound **10** was synthesized in a similar manner, starting from 6-phenylhexanoic acid and the alcohol *N*-{3-[(7-chloro-4-quinolyl)amino]-5-(hydroxymethyl)phenyl}-2-piperidinoacetamide (alcohol 8 in ref 41). The esters **2** and **3** were directly prepared from 6-[2-(3-methyl)naphthoquinolyl]hexanoic acid and the chloromethyl esters of the *n*-Pr or *t*-Bu acids according to described procedures.[42] The carbamate derivative **11** was synthesized following a three-step procedure: alcohol **8**[41] was activated by reaction with *N,N*′-disuccinimidyl carbonate, then the succinimidyl group was substituted by the 2-(5-aminopentyl)-3-methyl-1,4-naphthoquinone.

Stability Studies on the Esters. The stability of the prodrugs toward chemical hydrolysis in aqueous solu-

Table 1. In Vitro Sensitivity of Glutathione Reductase and of the *P. falciparum* Strain FcB1R[a] toward GR Inhibitors and the Prodrug Esters **1**−**3**



NQ	n	R_1	R_2	IC_{50} ($K_{i,c}$) for PfGR (μM)	IC_{50} ($K_{i,c}$) for hGR (μM)	ED_{50} FcB1R strain[a] (μM)
M_4	4	H	H	4.0 (3.1)	2.7 (1.8)	2.8 ± 1.1
M_5	5	H	H	0.5 (0.5)	2.6 (1.9)	3.5 ± 1.3
P_4	4	OH	H	2.5 (2.4)	1.0 (1.1)	7.7 ± 2.5
P_5	5	OH	H	1.3 (2.3)	0.75 (0.75)	8.2 ± 3.5
1	5	H	Me	na	na	10.8 ± 0.3
2	5	H		na	na	24.1 ± 2.5
3	5	H		na	na	22.4 ± 1.7

[a] The IC_{50} values represent the inhibition of the enzymes in standard assays using 100 μM GSSG. $K_{i,c}$ values (in parentheses) are calculated inhibitor constants assuming competitive inhibition. The esters **1**−**3** were prepared from the most efficient GR inhibitor **M_5**. As controls, ED_{50} values against the moderately CQ-resistant FcB1R strain were determined for CQ (126 ± 28 nM), isoalloxazine **3b**[36] (1.75 μM as mean of two independent experiments), and plumbagin (0.7 μM as mean of two independent experiments). The compounds listed here showed no cytotoxicity when tested at 2, 8, and 32 μM against hMRC-5 cells. na ≡ not active. Duplicate IC_{50} experiments yielded results differing by less than 6%.

Table 2. Susceptibility of Prodrug Esters **1**−**4**, **7**, and **8** to Hydrolysis at 37 °C under Quasi-physiologic Conditions[a]

	$t_{1/2}$ (h)		
esters	pH 5.0 acetate medium	pH 7.4 phosphate buffer	RPMI−10% human serum
1	0.5	1	>3
2	1	2	nd
3	0.5	1	nd
4	>80	>80	>80
7	>80	>80	>80
8	>80	>80	>80

[a] The $t_{1/2}$ for ester hydrolysis was determined by HPLC analysis (mean of two determinations). nd ≡ not determined.

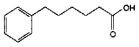
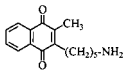
tions was evaluated under quasi-physiologic conditions by determining the half-life of ester disappearance (Table 2). pH 5.0 and pH 7.4 represent the milieu of the food vacuole and the cytosol of the parasite, respectively. The rates of enzymatic hydrolysis of the prodrugs were also determined in RPMI medium supplemented with 10% human serum because this is the medium commonly used for testing drugs in *P. falciparum* cultures. The non-amino esters **1**−**3** were highly susceptible to hydrolysis with half-lives below 2 h (Table 2). In contrast, the aminoesters **4**, **7**, and **8** were highly stable toward both chemical and extracellular hydrolysis with half-lives longer than 80 h (Table 2). By use of a linear regression treatment of the data, a $t_{1/2}$ value of 5 days was estimated for the most active ester **7** at pH 7.4 whereas the other double-drugs **4** and **8** were even more stable.

Inhibition of Heme Degradation. In the glutathione-dependent heme degradation assay, 52% heme degradation was observed within 10 min in the control sample containing 10 μM heme, 2 mM GSH, and 1 μL of DMSO. In the presence of 10 μM **M_5**, this degradation was not impaired at all, while 10 μM esters **7** and **8** inhibited the GSH-dependent heme degradation observed in the control by 40% and by 95%, respectively.

Biological Results

In Vitro Activity against the *P. falciparum* FcB1R Strain. Initially, the compounds were tested for their antimalarial activity against the moderately CQ-resistant parasite strain FcB1R (ED_{50} CQ = 126 nM). When tested for their in vitro activity upon the erythrocytic stage of *Plasmodium falciparum*, on the CQ-resistant strain FcB1R, the four compounds shown in Table 1 revealed a poor antimalarial activity (ED_{50} in the 3−8 μM range). This was expected because the presence of the carboxylate function is likely to prevent cell penetration. The prodrugs **1**−**3** showed an even lower antimalarial activity when compared with the starting carboxylic acid **M_5** (Table 1). In contrast, the double-drugs **4** and **6**−**9** prepared from the amino alcohols, hydroxychloroquine (hydroxyCQ), or the alcohol **8**[41] displayed potent antimalarial effects in the nanomolar range (Table 3). Ester **4** was found to be 2.4-fold more active than the parent alcohol, hydroxy-CQ. This was also true for esters **6**−**9** (ED_{50} = 23−56 nM) when compared with the starting alcohol **8**;[41] the most active ester **7** was prepared from the most efficient PfGR inhibitor (**M_5**). To ascertain the requirement for **M_5**, derivatives **5**, **10**, and **11** were synthesized as controls for the biological experiments. Both starting carboxylic acids (**M_2** and 6-phenylhexanoic acid) and 2-(5-amino-

Table 3. In Vitro Sensitivity of the *P. falciparum* FcB1R Strain toward the Antimalarial Alcohols Hydroxy-CQ or **8**[41] and to the Prodrug Esters **4** and **6**−**9**[a]

Compound	Starting Acid or Amine	Starting Alcohol	ED_{50} (nM) with FcB1R
-	-	HydroxyCQ	259 ± 24[f]
4	**M_5**	HydroxyCQ	107 ± 8[b]
-	-	alcohol **8**[41]	79.5 ± 18.3[d]
5	**M_2**	alcohol **8**[41]	144 ± 41[f]
6	**M_4**	alcohol **8**[41]	47 ± 5[b]
7	**M_5**	alcohol **8**[41]	23.1 ± 6.9[f]
8	**P_4**	alcohol **8**[41]	28.7 ± 5.5[b]
9	**P_5**	alcohol **8**[41]	56 ± 8[b]
10		alcohol **8**[41]	368 ± 45[b]
11		alcohol **8**[41]	144 ± 41[f]

[a] The compounds **5**, **10**, and **11** served as negative controls. The ED_{50} value for CQ toward the moderately CQ-resistant strain FcB1R is 126 ± 28 nM. Alcohol **8**[41] was used as the base form. The compounds were also tested for cytotoxic effects on hMRC-5 cells. The double-drug esters listed here showed no cytotoxicity when tested at 1.6, 6.3, and 25 μM; only the alcohol **8**[41] (100% at 25 μM but no effect at 6.3 μM) and ester **5** (90% at 25 μM, 23% at 6.3 μM, and 21% at 1.6 μM) were positive. [b] Number of experiments $n = 3$. [c] $n = 7$. [d] $n = 8$. [e] $n = 5$. [f] $n = 4$.

pentyl)-3-methyl-1,4-naphthoquinone, used to prepare **5**, **10**, and **11**, respectively, did not inhibit GRs, even when the side chain as in **10** and **11** had the same length as in the lead compound **M_5**. As expected, comparatively low antimalarial effects were observed for the three compounds. The most active compound ester **7** against the CQ-resistant strain FcB1R was subsequently evaluated for its efficiency in inhibiting the growth of six parasite strains that showed different degrees of CQ resistance (Table 4). In each case the ED_{50} of ester **7** was found to be approximately 28 nM, which indicates that it is equally effective against the six CQ-sensitive and CQ-resistant strains tested.

In Vitro Cytotoxicity upon hMRC-5 Cells. The starting alcohol **8**[41] is known to be cytotoxic both in vitro and in vivo, which has prevented further development in clinical trials.[41] When applying 25 μM alcohol **8**,[41] we observed 100% cytotoxicity with the human diploid embryonic lung cell line (hMRC-5, Bio-Whittaker 72211D). In contrast, 0% cytotoxicity was observed at 25 and 32 μM, with the five double-drugs **4** and **6**−**9** prepared from hydroxychloroquine or the alcohol **8**,[41] respectively (Table 3). In a further control, cytotoxicity upon hMRC-5 cells was observed for ester **5** already at 1.6 μM.

Total Glutathione, Glutathione Reductase, and Glutathione-*S*-Transferase in *P. falciparum* Incubated with 7. Ester **7** was incubated with ring stages of *P. falciparum* (CQ-resistant K1 strain) in order to determine its effects on different biochemical parameters at the subsequent trophozoite stage. The parasites in the ring stage were incubated with a single dose of 0, 30, 150, or 500 nM **7** for 24 h. Subsequently, the parasites of each sample were isolated and extracted. As demonstrated in Table 5, the protein content of the parasite culture clearly decreased with increasing concentration of the drug. Similar data were obtained for nucleic acids (data not shown). These observations are indicative of unspecific antiparasitic effects. However, when the nucleic acid content was corrected for protein, quite stable values were obtained for different drug concentrations (Table 5). Total glutathione content of the parasites was found to be slightly increased at the lowest inhibitor concentration and drastically decreased (below the detection limit of approximately 1 nmol/mg) at 150 and 500 nM of the inhibitor. Interestingly, GR activity (also corrected for protein) was found to be dose-dependently decreased and glutathione-*S*-transferase activity was increased.

In Vivo Antimalarial Activity upon *P. berghei*. Double-drugs **7** and **8** that had passed successfully all studies were subsequently tested for their antimalarial action in vivo using the murine malaria model infected with *P. berghei* (Table 6). Groups of three mice each were inoculated ip with infected cells. One group served as control. The other groups were treated with esters **7** and **8** or with the alcohol **8**[41] at 40 mg/kg. A 110% excess mean survival time (MST) was determined for the alcohol **8**.[41] Drug treatment was repeated for 4 consecutive days with daily counts of parasitemia. The mice were left after the end of treatment to check their survival. A decrease of more than 99.9% in parasitemia was observed with ester **7**, but the mice were not totally cured because they died from infection on days 23−25. Under the same conditions, untreated control mice and mice treated with the ester **8** died between days 7 and 10 after infection. From these data, 178% and 39% excess MST were determined for esters **7** and **8**, respectively. As an additional finding, no in vivo cytotoxicity was observed at 40 mg/kg for the ester **7**.

Discussion

The design and the synthesis of double-drugs allowed us to study the impact of GR inhibition, alone or in

Table 4. In vitro Efficiency of the Alcohol **8**[41] and the Double-Drug Ester **7** against Parasite Strains Exhibiting Different Degrees of Resistance to CQ[a]

compound	strain THAI ED_{50} (nM)	strain F32 ED_{50} (nM)	strain D6 ED_{50} (nM)	strain FcB1 ED_{50} (nM)	strain W2 ED_{50} (nM)	strain K1 ED_{50} (nM)
CQ	14.3 ± 2.4[b]	19 ± 4[b]	54 ± 12[b]	126 ± 28[b]	175 ± 31[b]	183 ± 35[b]
alcohol **8**[41]	89.6 ± 13.1[b]	42.8 ± 8.2[c]	55.2 ± 5.2[b]	79.5 ± 18.3[d]	78 ± 14.8[e]	167.7 ± 34.7[e]
ester **7**	36.5 ± 3.3[b]	25.8 ± 6.5[b]	27.7 ± 3.2[b]	23.1 ± 6.9[f]	26.4 ± 6.2[b]	29.3 ± 2.5[b]

[a] Parasites were considered resistant to CQ when ED_{50} was >100 nM. ED_{50} values are the mean ± standard deviation of at least three independent experiments. Alcohol **8**[41] was used as the base form. [b] Number of experiments $n = 3$. [c] $n = 7$. [d] $n = 8$. [e] $n = 5$. [f] $n = 4$.

Table 5. Effects of Double-Drug **7** on Protein, Nucleic Acid, and Glutathione Content, as Well as on Glutathione Reductase (GR) and Glutathione-*S*-Transferase (GST) Activities of the Chloroquine Resistant *P. falciparum* Strain K1 in Culture

treatment for 24 h[a]	protein (mg/mL)	nucleic acids[b] (ng/mg)	glutathione[b] (nmol/mg)	GR activity[b] (mU/mg)	GST activity[b] (mU/mg)
none	1.2	86	10.8	30	1.7
30 nM **7**	1.1	89	14.8	30	3.7
150 nM **7**	0.6	79	<1.0[c]	8	4.3
500 nM **7**	0.4	81	<1.0[c]	6	9.5

[a] For details see text. [b] Values in columns 3−6 refer to 1 mg of protein in the extract (see column 2). All values represent mean values of three single determinations that differed by less than 10%. [c] The detection limit was 1.0 nmol/mg.

Table 6. In Vivo Efficiency of the Double-Drugs **7** and **8** in Mice Infected with *P. berghei*

treatment[a]	parasitaemia (%) day 4	parasitaemia (%) day 11	parasitaemia (%) day 18	death	% excess MST[b]
none	20			before day 11	
ester **7**	<0.1	9	48	on days 23−25	178
ester **8**	9			before day 11	39

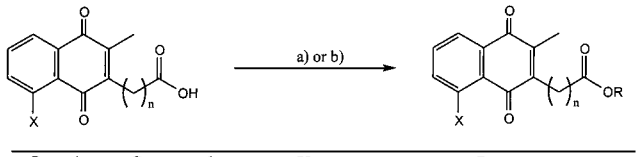
[a] Infection on day 0, intraperitoneal treatment with 40 mg compound/kg on days 1−4 (1 dose per day). [b] A 110% excess MST was found for the alcohol **8**[41] when used as the chlorohydrate form.

association with the effects of 4-aminoquinolines, on *Plasmodium* species in vitro and in vivo. As a first step, a series of carboxylic acids were identified as potent reversible GR inhibitors by screening a library of 1400 1,4-naphthoquinone derivatives. The superiority of the alcanoic acids over other NQ compounds can be explained by their competition with the anionic substrate glutatione disulfide for a positively charged binding site on the enzyme.[43] The four most active GR inhibitors, $\mathbf{M_4}$, $\mathbf{M_5}$, $\mathbf{P_4}$, and $\mathbf{P_5}$, displayed only moderate effects on a *P. falciparum* FcB1R culture (Table 1) probably because the negative charge prevents these compounds from crossing cellular membranes. Consequently, we masked the carboxylate groups by preparing a number of esters with different chemical and biological properties as prodrugs (compounds **1**−**9**). Expectedly none of them exhibited significant GR inhibition when compared to the parent alcanoic acids in the standard enzyme assay.

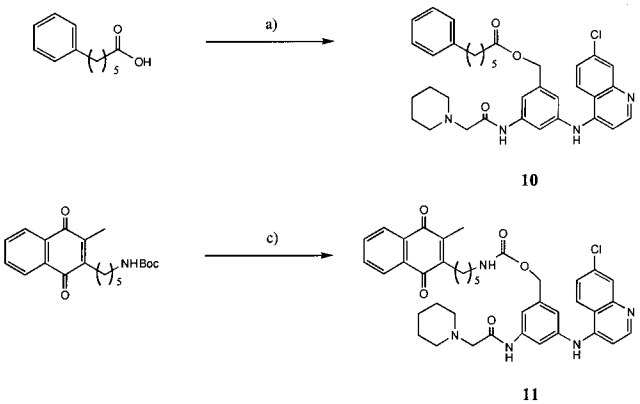
The first group of prodrugs (compounds **1**−**3**) comprised labile esters that were prepared by acylation of aliphatic non-amino alcohols. These esters had disappointingly low activities against *P. falciparum* in culture (Table 3), probably because hydrolysis rates in the culture medium were too high, which led to premature release of the GR inhibitor (Table 2).

To improve the pharmacokinetic properties, a second group of esters was designed using the 4-aminoquinoline-containing alcohols, hydroxy-CQ, or compound **8**.[41] These amino alcohols are known to exert antiplasmodial activity on their own merits (Scheme 1 and Table 3). Consequently esters **4** and **6**−**9** were called double-headed prodrugs or double-drugs because both the NQ alcanoic acid and the alcoholic component have antimalarial activity. Their targets in the parasite, GSSG reduction and heme detoxification metabolism, respectively, are different but functionally linked biochemical pathways. The most active compound in vitro and in vivo was the aminoester **7** (the condensation product of $\mathbf{M_5}$ and the 4-anilinoquinoline-based alcohol **8**[41]), and

Scheme 1. Synthesis of the Esters **1**−**10** and Carbamate **11**[a]



Reaction conditions	Compound	n	X	R
a	**1**	5	H	Me
b	**2**	5	H	
b	**3**	5	H	
a	**4**	5	H	
a	**5**	2	H	
a	**6**	4	H	
a	**7**	5	H	
a	**8**	4	OH	
a	**9**	5	OH	



[a] Reaction conditions: (a) ROH/DCC/DMAP/CH_2Cl_2; (b) RCO_2-CH_2Cl/Et_3N/DMF; (c) (i) HCl 1 N, MeOH, (ii) alcohol **8**,[41] DSC, CH_2Cl_2, pyridine, 12 h, then condensation step in DMF.

the following discussion largely refers to this compound. Ester **7** was found to be equally effective against CQ-sensitive and CQ-resistant *P. falciparum* strains, the EC_{50} value being below 30 nM in all but one case (Table 4). In addition, treatment with ester **7** led to a highly significant increase of survival time (from 8 to 24 days) in *P. berghei* infected mice (Table 6).

Apart from its own pharmacologic effects, the amino alcohol component was meant to confer the following

properties to ester **7** and related prodrugs. (i) As a CQ-like 4-aminoquinoline, it leads to accumulation of the ester in the parasite's food vacuole. (ii) It provides an ester bond that is sufficiently stable in biological compartments such as blood plasma and cytosolic spaces and when permeating the membranes of parasitized erythroytes. This ester bond, however, is subject to enzymic hydrolysis at the low pH (5.5) of the food vacuole, which contains numerous esterases, proteases, and other hydrolases for the degradation of host cytoplasmic components. From the acidic food vacuole where the released alcanoic acid $\mathbf{M_5}$ is partially protonated, the transition to the parasite's cytosol appears to be but a minor obstacle. It is here where the alcanoic acid is assumed to meet its target glutathione reductase. The amino alcohol, in contrast, has targets both in the food vacuole where it prevents growth of the hemozoin polymer and in the cytoplasma where it interferes with heme−glutathione interaction.[3,8]

Of course, we cannot exclude the possibility that a substantial portion of the double-headed prodrug is directly hydrolyzed in the parasite's cytosol. In this context it is significant that the double-drug ester **7** was found to be nontoxic to the human cell line hMRC-5, which contrasts with the cytotoxicity of the starting alcohol **8**.[41] These observations suggest that ester **7** does not accumulate and is not hydrolyzed in parasite-free human cells. Also, in the therapy studies using ester **7** in mice (Table 6), this compound did not show any obvious signs of mammalian toxicity.

We also conducted experiments on the putative pharmacologic mechanism of action. The prodrug **7** led to a dose-dependent decrease in total glutathione content and in GR activity of malarial parasites in culture (Table 5). These effects were not observed in vitro in the GR assay for the alcohol **8**,[41] which makes it very likely that the low glutathione level is due to the release of the GR inhibitor $\mathbf{M_5}$ from the prodrug ester in situ; it is known that insufficient GSSG reduction activity leads to GSSG accumulation at the expense of GSH and to the export of GSSG from the parasite.[44] In contrast, the low GR activities found in the parasite extracts cannot be ascribed to the reversible inhibition of GR by $\mathbf{M_5}$ in situ because the GR−$\mathbf{M_5}$ complex would dissociate under the conditions of extraction and subsequent enzyme assaying. A speculative explanation for the low extractable activity is that GR forms a ternary complex with $\mathbf{M_5}$ and its reducing substrate NADPH in situ; in this complex the enzyme's structure is probably destabilized and readily degraded.[45] In vitro at 37 °C, this ternary complex has a half-life of 6 min at 0.1 μM protein concentration (Müssigbrodt, unpublished data).

The dose-dependent induction of GSH transferase (GST) activity in ester **7** treated parasites (Table 6) may be indicative of the parasites's adaptation to this compound but possibly also to the resultant increase of the intracytosolic heme level. This interpretation would be consistent with the studies of Dubois et al.[46] on the glutathione metabolism in CQ-resistant *P. berghei*. If the increased GST activity contributes to decreased GSH levels under conditions of GR inhibition and oxidative stress, the induction of GST would add to the potential therapeutic value of ester **7** as an antimalarial agent per se but also as a chloroquine sensitizer.

In conclusion, the efficiency both in vitro and in vivo of the double-drugs prepared from a GR inhibitor and a drug affecting heme metabolism appears to be a promising strategy for the development of new antimalarial double-drugs. Future developments on the basis of this model include other enzyme inhibitors linked to 4-aminoquinolines. Apart from GR inactivators, inhibitors of the enzymes involved in glutathione synthesis and of thioredoxin reductase are of interest as antiplasmodial principles per se but they can also serve as chloroquin sensitizers. *P. falciparum* thioredoxin reductase as a drug target has been highlighted in recent studies, which suggest that the thioredoxin system is largely responsible for the residual GSSG-reducing capacity observed after GR inhibition.[47]

Experimental Section

Abbreviations. CQ, chloroquine; DCC, dicyclohexylcarbodiimide; DMAP, 4-(dimethylamino)pyridine; DMSO, dimethyl sulfoxide; DSC, *N*,*N*′-disuccinimidyl carbonate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); GR, glutathione reductase; hGR, human glutathione reductase; PfGR, *Plasmodium falciparum* glutathione reductase; GSH, reduced glutathione; GSSG, glutathione disulfide; HPLC t_R, HPLC retention time; HTS, high-throughput screening; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; NQ, 1,4-naphthoquinone; Trx, thioredoxin; TrxR, thioredoxin reductase; hTrxR, human thioredoxin reductase.

Enzymes. Recombinant human and *Plasmodium falciparum* glutathione reductases (GR, EC 1.6.4.2) were purified as previously reported.[33,48] One unit of GR activity is defined as the consumption of 1 μmol of NADPH per min (ϵ_{340} nm = 6.22 mM^{-1} cm^{-1}) under conditions of substrate saturation. The enzyme stock solutions used for kinetic determinations were pure as judged from a silver-stained SDS-PAGE and had a specific activity of 200 U/mg (hGR) and of 100 U/mg (PfGR) in the GSSG assay, respectively. All other reagents were of the highest available purity and were purchased from Biomol, Boehringer, and Sigma.

GR Inhibitor Screening and GR Inhibition Studies. Automated HTS of the library was performed on a Beckman Biomeck 2000 apparatus, as previously described, by using the screening mixture (25 μM inhibitor, 200 μM GSSG, 500 μM NADPH, and 1% DMSO in 100 mM sodium phosphate buffer, 2 mM EDTA, pH 7.0).[37] Glutathione reductase activity was assayed at 25 °C with 100 μM NADPH and 100 μM GSSG in assay buffer (47 mM potassium phosphate, 200 mM KCl, 1 mM EDTA, pH 6.9). The consumption of NADPH was followed spectrophotometrically at 340 nm.[48] For inhibitor studies the assay containing NADPH, enzyme (approximately 5 mU/ml), and inhibitor was started with GSSG. IC_{50} values were evaluated in duplicate in the presence of increasing inhibitor concentrations and 1% DMSO as final concentration. The type of inhibition and inhibition constants were determined at 25 °C in duplicate experiments at six different inhibitor concentrations and four concentrations of GSSG (0.04, 0.1, 0.3, and 1 mM) again in the presence of 1% DMSO. For calculating inhibition constants ($K_{i,c}$) from observed enzyme activities in the absence (V_{max}) and the presence (v_i) of the inhibitor, we applied the equation for competitive inhibition:

$$\frac{V_{max}}{v_i} = 1 + \left(\frac{K_m}{[GSSG]}\right)\left(1 + \frac{[I]}{K_{i,c}}\right)$$

Stability Studies in Aqueous Solutions. The rates of chemical hydrolyses of prodrugs **1**−**4**, **7**, and **8** were studied at 37 °C in aqueous phosphate buffer of pH 7.4 (150 mM NaCl, 4 mM NaH_2PO_4, 25 mM $NaHCO_3$) and in aqueous acetate solution of pH 5.0 (150 mM NaCl, 10 mM NaOAc). A 1 mM stock solution of prodrug in 10% aqueous DMSO was diluted in preheated buffer, and the 100 μM solutions of prodrugs were

placed in a thermostatically controlled water bath at 37 °C. The 400 μL samples of the solutions were collected at different times (t = 0, 0.5, 1, 2, 3, 6, 20, 24, 30.5, 45, 54, 69.5, and 77.5 h). The 100 μL samples were added to 100 μL of EtOH and supplemented with 50 μL of internal reference and then were directly injected through a 50 μL loop for HPLC analysis. Analytical HPLC was performed on a Shimadzu system equipped with a C18 Macherey Nagel Nucleosil column (4 mm × 300 mm, 100 μm, 300 Å) and a UV detector set at 254 nm. The following solvent systems were used: (A), 0.05% trifluoroacetic acid (TFA) in H_2O and (B) 0.05% TFA, 20% H_2O, 80% CH_3CN, with the following conditions, depending on the esters: conditions I, (A) 5 min, a linear gradient from (A) to (B), 0−100% for 30 min, (A) 2 min; conditions II, (A) 1 min, a linear gradient from (A) to (B), 0−100% for 34 min, (A) 5 min. HPLC retention times (HPLC t_R) were obtained at flow rates of 1 mL/min. At pH 5.0, t_R = 15.0 min (conditions I) and 11.8 min (conditions II) for the internal reference, t_R = 26.0 min (conditions I) and 24.2 min (conditions II) for **M$_5$**, t_R = 23.5 min for **P$_4$** (conditions II), t_R = 29.5 min for **1** (conditions I), t_R = 13.8 min for the alcohol **8**[41] (conditions II), t_R = 16.5 min for hydroxy-CQ (conditions I), t_R = 23.7 min for **4** (conditions I), t_R = 22.5 min for **7** (conditions II), t_R = 22.0 min for **8** (conditions II). At pH 7.4, t_R = 15.0 min (conditions I) and 11.8 min (conditions II) for the internal reference, t_R = 29.5 min (conditions I) and 24.2 min (conditions II) for **M$_5$**, t_R = 23.6 min for **P$_4$** (conditions II), t_R = 29.5 min for **1** (conditions I), t_R = 13.6 min for the alcohol **8**[41] (conditions II), t_R = 16.5 min for hydroxy-CQ (conditions I), t_R = 23.7 min for **4** (conditions I), t_R = 22.5 min for **7** (conditions II), t_R = 22.1 min for **8** (conditions II). In the case of esters **2** and **3** only, a C18 TSKgel Super ODS column (4.6 mm × 50 mm, 110 Å) was used under the following conditions (conditions III): a linear gradient from (A) to (B), 0−100% (B), 10 min. At pH 5.0, t_R = 7.2 min for **2** (conditions III), t_R = 9.1 min for **3** (conditions III). On the basis of the peak area (in percent), the amounts of native compounds were determined from their disappearance by referring to the peak of (*N*-{3-[(7-chloro-4-quinolyl)amino]-5-[(*N*-{2-dimethylaminoethyl}-*N*-methylamino)methyl]phenyl}-2-piperidinoacetamide introduced as an internal reference (compounds **40** in ref 41).

Stability Studies in RPMI−10% Human Serum. The rates of enzymatic hydrolysis of esters **1**, **4**, **7**, and **8** were studied in RPMI−10% human serum at 37 °C. The reactions were initiated by dissolving a 1 mM stock solution of prodrug in 10% aqueous DMSO in the preheated medium, and the 100 μM solutions of prodrugs were placed in a thermostatically controlled water bath at 37 °C. The 400 μL samples of the solutions were collected at different times (t = 0, 0.5, 1, 2, 3, 6, 20, 24, 30.5, 45, 54, 69.5, and 77.5 h). The 100 μL samples were added to 100 μL of EtOH and supplemented with 50 μL of internal reference, then were centrifuged for 10 min at 8000 rpm. The supernatant was directly injected through a 50 μL loop in the HPLC apparatus described above. In the presence of human serum, t_R = 15.0 min (conditions I) and 11.8 min (conditions II) for the internal reference, t_R = 29.5 min (conditions I) and 24.2 min (conditions II) for **M$_5$**, t_R = 23.6 min for **P$_4$** (conditions II), t_R = 33.3 min for **1** (conditions I), t_R = 13.9 min for the alcohol **8**[41] (conditions II), t_R = 16.5 min for hydroxy-CQ (conditions I), t_R = 23.7 min for **4** (conditions I), t_R = 22.2 min for **8** (conditions II), t_R = 22.5 min for **7** (conditions II). On the basis of the peak area (in percent), the amounts of native compounds were determined from their disappearance by referring to the peak of (*N*-{3-[(7-chloro-4-quinolyl)amino]-5-[(*N*-{2-dimethylaminoethyl}-*N*-methylamino)methyl]phenyl}-2-piperidinoacetamide introduced as an internal reference (compound **40** in ref 41).

Glutathione-Dependent Heme Degradation. Heme modification by GSH was studied according to Atamna and Ginsburg[4] with slight modifications. A 1 mM heme (Porphyrin Products, Inc., Logan, UT) in 0.1 M NaOH and 400 mM GSH in water were prepared freshly prior to use. Heme was diluted to a final concentration of 10 μM in 200 mM Hepes, pH 7.0, and the reaction was started by adding GSH to a final concentration of 2 mM. Spectral changes between 300 and 500 nm were measured immediately after mixing and then at 60 s intervals. For studying the effects of drugs on degradation, 1 μL of a 10 mM drug solution in DMSO was added, 10 μM was added to the buffer containing 10 μM heme, and the reaction was started with GSH. The rate of heme degradation was determined on the basis of absorbance decrease at 390 nm.

Biological Assays. 1. *P. falciparum* Cultivation and Drug Assays. *P. falciparum* strains were maintained in a continuous culture of human erythrocytes as described by Trager and Jensen.[49] In vitro antiplasmodial activity of our compounds was determined using a modification of the semiautomated microdilution technique of Desjardins et al.[50] CQ-sensitive *P. falciparum* strains (Thai/Thailand, F32/Tanzania, D6/Sierra-Leone) and CQ-resistant strains (FcB1R/Colombia, W2/Indochina, K1/Thailand) were used for sensitivity testing. FcB1R, F32 were strains obtained by limit dilution. Stock solutions of chloroquine diphosphate and tested compounds were prepared in sterile distilled water and DMSO, respectively. Drug solutions were serially diluted with culture medium and added to asynchronous parasite cultures (0.5% parasitemia and 1% final hematocrit) in 96-well plates. After 24 h at 37 °C, we added 0.5 μCi of [^{3}H]hypoxanthine (1−5 Ci/mmol; Amersham, Les Ulis, France) *per* well, and the cultures were left for another 24 h at 37 °C. The growth inhibition for each drug concentration was determined by comparison of the radioactivity incorporated in the treated culture with that in the control culture (without drug) maintained on the same plate. The drug concentration causing 50% inhibition (IC_{50}) was obtained from the concentration−response curve, and the results were expressed as the mean determined from several independent experiments. The DMSO introduced into the cultures never exceeded 0.1% and did not affect parasite growth.

2. Cytotoxicity Test upon hMRC-5 Cells. A human diploid embryonic lung cell line (MRC-5, Bio-Whittaker 72211D) and primary peritoneal mouse macrophages were used to assess the cytotoxicity for host cells. The macrophages were collected from the peritoneal cavity 48 h after stimulation with potato starch and seeded into 96-well microplates at 30 000 cells *per* well. The hMRC-5 cells were seeded at 5000 cells *per* well. After 24 h, the cells were washed and 2-fold dilutions of the drug were added to 200 μL of standard culture medium (RPMI + 5% fetal calf serum). The final DMSO concentration in the culture remained below 0.5%, which had no apparent effect per se. The cultures were incubated with four concentrations of the compounds (25, 12.5, 6.25, and 3.13 μM or 32, 8, 2, and 0.5 μM) for 7 days at 37 °C in 5% CO_2−95% air. Untreated cultures were included as controls. For MRC-5 cells, the cytotoxicity was determined using the colorimetric MTT assay[51] and scored as percentage of absorbance reduction at 540 nm of treated cultures versus untreated control cultures. For macrophages, viability assessment was performed microscopically.

3. Total Glutathione, Protein, and Nucleic Acid Content as Well as GR and GST Activity of *P. falciparum* Incubated with Ester 7. Intraerythrocytic stages of the CQ-resistant *P. falciparum* strain K1 were cultured in vitro according to Trager and Jensen[49] and synchronized with sorbitol. A 10 mM stock solution of **7** was prepared in PBS prior to use and diluted in medium. Synchronized cultures at the ring stage (30 mL, 5% hematocrit, 3.7% parasitemia) were treated with the drug at a final concentration of 0, 30, 150, and 500 nM, respectively, and incubated for 24 h. Parasite isolation was achieved by suspending the red cells in a 20-fold volume of buffer containing 7 mM K_2HPO_4, 1 mM NaH_2PO_4, 11 mM $NaHCO_3$, 58 mM KCl, 56 mM NaCl, 1 mM $MgCl_2$, 14 mM glucose, and 0.02% saponin for 10 min at 37 °C. The pellets were washed three times, and the parasites were diluted in 150 μL of the same buffer and disrupted three times by freezing and thawing. After centrifugation, the supernatant was used for the various analyses.

Different volumes of parasite extract were diluted in 1 mL of 150 mM KH_2PO_4, pH 8.0, and on the basis of the absorbance at 260 and 280 nm, nucleic acid and protein content was determined. Protein content was, in addition, measured by the BioRad protein dye assay (with BSA as a standard). For the determination of total glutathione content, 40 μL of parasite extract was deproteinized by adding 2 volumes of 5% sulfosalicylic acid; the samples were mixed and centrifuged, and the supernatant was stored at −20 °C. The GSH content was measured by the GR-coupled DTNB−GSH−recycling assay.[52] A standard curve was prepared using appropriate concentrations of GSH and sulfosalicylic acid. GR activity in parasite extracts was measured as described above. GST activity was determined spectrophotometrically according to Beutler[53] with 0.5 mM CDNB and 1 mM GSH in 100 mM K_2HPO_4/KH_2PO_4, 1 mM EDTA, pH 6.5 at 340 nm.

4. In Vivo Activity against *P. berghei*. In vivo antimalarial activities were determined in mice infected with *P. berghei* (ANKA 65 strain). Four-week-old female Swiss mice (CD-1, 20−25 g) were intraperitoneally infected with about 10^7 parasitized erythrocytes, collected from the blood of an acutely infected donor animal. At the same time, the animals (three animals *per* group) were treated *per os* with the drug tested at 40 mg/kg (drug formulation in 100% DMSO). The treatment was continued over the following 4 days by intraperitoneal injection. Untreated control infected mice generally die between 7 and 10 days following infection. Drug activity was evaluated as the prolongation of the percentage of excess mean survival time (MST) observed with untreated controls. Three infected control animals received only DMSO injections.

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Supporting Information Available: Details of chemical procedures and analytical data of compounds **1**−**11**, Table 1 showing the inhibition of GR from Man and *Plasmodium falciparum* by carboxylic acids of the menadione, plumbagin, and juglone series, Figure 1 showing the inhibition of *P. falciparum* GR by 6-[2-(3-methyl)naphthoquinolyl]hexanoic acid (**M$_5$**). This material is available free of charge via the Internet at http://pubs.acs.org.

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