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A Fluoro Analogue of the Menadione Derivative 6-[2′-(3′-Methyl)-1′,4′-naphthoquinolyl]hexanoic Acid Is a Suicide Substrate of Glutathione Reductase. Crystal Structure of the Alkylated Human Enzyme†

Holger Bauer,[‡,§] Karin Fritz-Wolf,[||] Andreas Winzer,[‡] Sebastian Kühner,[‡] Susan Little,[⊥] Vanessa Yardley,[⊥] Hervé Vezin,[⊗] Bruce Palfey,[○] R. Heiner Schirmer,[‡] and Elisabeth Davioud-Charvet*[,‡,§]

Contribution from the Biochemie-Zentrum der Universität Heidelberg, Im Neuenheimer Feld 504, D-69120 Heidelberg, Germany, Centre National de la Recherche Scientifique, 3 rue Michel-Ange, 75794 Paris cedex 16, France, Max-Planck-Institut für Medizinische Forschung, Jahnstrasse 29, D-69120 Heidelberg, Germany, Department of Infections and Tropical Diseases, London School of Hygiene & Tropical Medicine, Keppel Street, London WC1E 7HT, United Kingdom, Laboratoire de Chimie Organique et Macromoléculaire, UMR-CNRS 8009, Bât. C4, Université des Sciences et Technologies de Lille, 59655 Villeneuve d'Ascq cedex, France, and Department of Biological Chemistry, The University of Michigan, Ann Arbor, Michigan 48109-0606

Received February 27, 2006; E-mail: elisabeth.davioud@gmx.de

Abstract: Glutathione reductase is an important housekeeping enzyme for redox homeostasis both in human cells and in the causative agent of tropical malaria, *Plasmodium falciparum*. Glutathione reductase inhibitors were shown to have anticancer and antimalarial activity per se and to contribute to the reversal of drug resistance. The development of menadione chemistry has led to the selection of 6-[2′-(3′-methyl)-1′,4′-naphthoquinolyl]hexanoic acid, called M_5, as a potent reversible and uncompetitive inhibitor of both human and *P. falciparum* glutathione reductases. Here we describe the synthesis and kinetic characterization of a fluoromethyl-M_5 analogue that acts as a mechanism-based inhibitor of both enzymes. In the course of enzymatic catalysis, the suicide substrate is activated by one- or two-electron reduction, and then a highly reactive quinone methide is generated upon elimination of the fluorine. Accordingly the human enzyme was found to be irreversibly inactivated with a k_{inact} value of 0.4 ± 0.2 min^{-1}. The crystal structure of the alkylated enzyme was solved at 1.7 Å resolution. It showed the inhibitor to bind covalently to the active site Cys58 and to interact noncovalently with His467′, Arg347, Arg37, and Tyr114. On the basis of the crystal structure of the inactivated human enzyme and stopped-flow kinetic studies with two- and four-electron-reduced forms of the unreacted *P. falciparum* enzyme, a mechanism is proposed which explains naphthoquinone reduction at the flavin of glutathione reductase.

Introduction

The homodimeric FAD-containing glutathione reductase (GR; EC 1.8.1.7) belongs to the family of NADPH-dependent oxidoreductases and is present in many pro- and eukaryotic organisms. GR reduces glutathione disulfide (GSSG) to its thiol form GSH:

$$GSSG + NADPH + H^+ \rightarrow 2\,GSH + NADP^+$$

The catalytic mechanism of GR from various sources has been investigated by Williams et al. for many decades.[1] The flow of electrons during GR catalysis is from NADPH to the *re* face of the flavin (FAD) and then from the *si* face of FAD to the active center disulfide ($C^{58}VNVGC^{63}$, numbering referring to human GR) and hence to GSSG. Although the enzyme can accept four electrons per subunit (two at the flavin and two at the disulfide), the principal catalytically active forms are the oxidized E_{ox} and the two-electron-reduced EH_2 forms (Scheme 1). In the EH_2 state the active site disulfide is reduced while

† Abbreviations: BPO, benzoyl peroxide; BuLi, butyllithium; CAN, cerium(IV) ammonium nitrate; CI-MS, chemical ionization mass spectrometry; DAST, *N*,*N*-diethylaminosulfur trifluoride; DMSO, dimethyl sulfoxide; E_{ox}, enzyme with the flavin and redox-active disulfide in the oxidized state; EH_2, two-electron-reduced enzyme with the redox-active dithiol; EH_4, four-electron-reduced enzyme with the reduced flavin and the redox-active dithiol; EI-MS, electron ionization mass spectrometry; FAD, flavin; GR, glutathione reductase; GSH, reduced glutathione; GSSG, glutathione disulfide; NBS, *N*-bromosuccinimide; NQ, naphthoquinone; SCE, standard calomel electrode; TBAF, tetrabutylammonium fluoride.

‡ Biochemie-Zentrum der Universität Heidelberg.
§ Centre National de la Recherche Scientifique.
|| Max-Planck-Institut für Medizinische Forschung.
⊥ London School of Hygiene & Tropical Medicine.
⊗ Université des Sciences et Technologies de Lille.
○ The University of Michigan.

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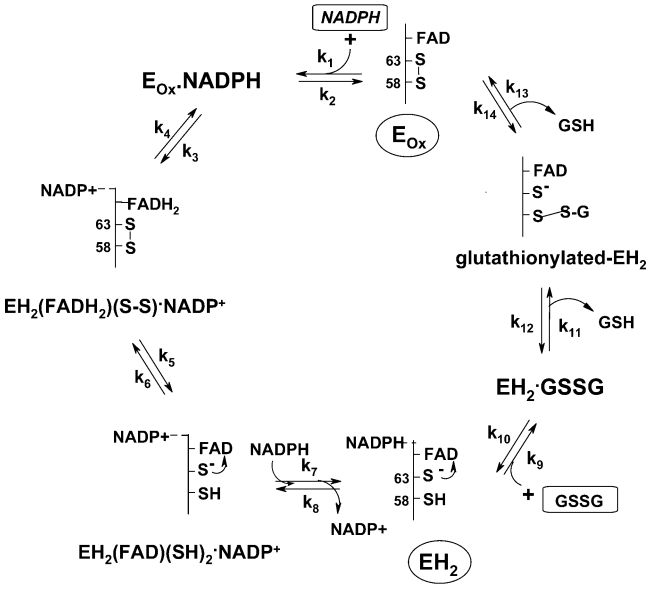
Scheme 1. Catalytic Cycle of Human Glutathione Reductase



the FAD has been oxidized. Catalysis[2−4] involves electrons transferred from NADPH to flavin to form $FADH_2$, and the reduced FAD very rapidly transfers electrons to the disulfide center (Scheme 1). In the EH_2 enzyme a charge-transfer interaction is formed between the proximal thiolate Cys63 as the donor and the flavin as the acceptor. After $NADP^+$ release GSSG is reduced in the oxidative half-reaction. The solvent-exposed thiol in EH_2, Cys58, initiates the dithiol−disulfide interchange with GSSG that leads to an *S*-glutathionylated enzyme and the generation of the first molecule of glutathione. Then the second thiol Cys63 attacks the mixed disulfide bridge of the glutathionylated enzyme, releasing the second molecule of glutathione and re-forming the oxidized enzyme E_{ox}.

Glutathione is a major player in intracellular redox homeostasis and in detoxication processes both in human cells and in the malarial parasite *Plasmodium falciparum*.[5−7] Plasmodia are sensitive to disturbances of the redox metabolism. This fact is of special importance during the intraerythrocytic life stage where the parasites are exposed to an increased oxidative stress level.[8] It was observed in parasite cultures with different *P. falciparum* strains that the degree of chloroquine resistance is directly linked to the levels of reduced glutathione.[9−11] The importance of glutathione in this field was further substantiated by the finding that a decrease of the glutathione level in rodent malaria leads to a potentiation of the antimalarial action of chloroquine.[12]

Our present research aims at the design of inhibitors of GR, which has been identified as a gate keeper protein in the glutathione redox system. Glutathione reductase inhibitors are usually considered as drug sensitizers: they do not display high intrinsic antimalarial or antitumoral activities when used alone, but they can enhance the effects of chloroquine or cytotoxic agents. By high-throughput screening, it was found that a carboxylic acid derivative of menadione, M_5 (6-[2′-(3′-methyl)-1′,4′-naphthoquinolyl]hexanoic acid), is a potent inhibitor of *P. falciparum* GR in the low micromolar range.[13] Double-headed prodrugs based on M_5 showed potent antimalarial activities both in vitro and in vivo in the mouse model.[13] M_5 is uncompetitive with respect to both NADPH and GSSG.[14] The binding site of the inhibitor could, however, not be elucidated so far, which suggests that it binds to an enzyme−substrate intermediate that occurs in the catalytic cycle (Scheme 1).

Naphthoquinone derivatives are also substrates of flavoprotein disulfide reductases.[15,16] This finding was our motivation to design a suicide substrate, or mechanism-based inhibitor of GR. With a leaving group present in the position β to the carbonyl group of the quinone core, the system can be activated by one- or two-electron reduction, and a highly reactive quinone methide is formed after release of the leaving group (Scheme 2).[17] This intermediate is expected to react easily with nucleophilic residues in the vicinity.[18] We introduced a fluorine atom β to the carbonyl group of menadione as an almost isosteric replacement of hydrogen. Further advantages are that the carbon−fluorine bond is the most stable of the carbon−halogen bonds and fluorine is meant to support the postulated mechanism by formation of an intramolecular hydrogen bond (Scheme 2).[17] Two fluoromethyl analogues of M_5 were synthesized. Time-dependent inhibition of both GR from human and *P. falciparum* was observed using the GSSG reduction assay and studied. The alkylated human GR was crystallized, and the 3D structure was resolved at 1.7 Å resolution. The potential binding and reduction site(s) of naphthoquinone can be discussed in light of the observed kinetics using the inactivated human enzyme or two- and four-electron-reduced forms of the unreacted *P. falciparum* enzyme, the electrochemical behavior of the newly synthesized compounds, and the 3D structure of the human GR alkylated by 6-[2′-(3′-fluoromethyl)-1′,4′-naphthoquinolyl]hexanoic acid (fluoro-M_5).

Experimental Section

Glutathione Disulfide Reductase Assays. The standard assay was conducted at 25 °C in a 1 mL cuvette. The assay mixture contained 100 μM NADPH and 1 mM GSSG in GR buffer (100 mM potassium phosphate buffer, 200 mM KCl, 1 mM EDTA, pH 6.9). IC_{50} values were evaluated in duplicate in the presence of seven inhibitor concentrations ranging from 0 to 100 μM. Inhibitor stock solutions

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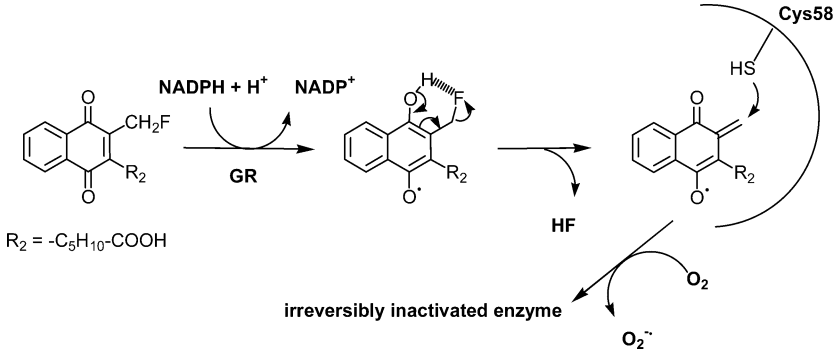
Scheme 2. Proposed Mechanism of the Irreversible Inactivation of Glutathione Reductase by Fluoro-M_5[a]



[a] The naphthoquinone is activated via GR-catalyzed one-electron reduction (two-electron transfer is also possible). Elimination of HF promoted by a newly formed intramolecular hydrogen bond results in a quinone methide. Nucleophilic attack of the catalytic Cys58 leads to covalent modification of the enzyme. The radical can further react with oxygen, leading to the formation of superoxide.

were prepared in 100% DMSO. A 1% final DMSO concentration was kept constant in the assay cuvette. The reaction was started by adding enzyme (8 mU of human GR, 6.5 mU of *P. falciparum* GR), and initial rates of NADPH oxidation were monitored at 340 nm ($\epsilon_{340\ nm} = 6.22\ mM^{-1}\ cm^{-1}$).

Time-Dependent Inactivation of Glutathione Reductases by Fluoro-M_5. For determining the rate constants of human and *P. falciparum* GR inactivation, the residual GR activity was monitored over time by following an incubation protocol.[19] All reaction mixtures (final volume of 50 μL) contained 160 μM NADPH, varying inhibitor concentrations (0−5 μM in the human GR assay, 0−50 μM in the *P. falciparum* GR assay), GR (6.85 pmol of human GR, 21 pmol of *P. falciparum* GR), and 2% DMSO in GR buffer at 25 °C. At different time points, 5 μL aliquots of each reaction mixture were removed and the residual activity was measured in the standard GSSG reduction assay at 25 °C (1 mM GSSG and 100 μM NADPH). DMSO (2%) was used in control assays.

Menadione Reductase Activity of Human and *P. falciparum* Alkylated Glutathione Reductases. The ability of alkylated GRs to reduce menadione was assayed by monitoring the oxidation of NADPH at 340 nm ($\epsilon_{340\ nm} = 6.22\ mM^{-1}\ cm^{-1}$). Over a 2 h incubation period 300 μM fluoro-M_5 was allowed to react with 86 μM human GR (891 μg) in a 198 μL total volume containing 400 μM NADPH at 25 °C prior to the naphthoquinone reduction assay. A similar alkylation experiment was done with 72 μM *P. falciparum* GR (1.06 mg) over a 15 h long incubation time in a total volume of 257 μL, due to gradual addition of NADPH (final 780 μM) and the alkylating agent (final 690 μM fluoro-M_5). At different time periods, residual GSSG reduction ability was tested in the standard GSSG reduction assay to check alkylation completion. When total enzyme alkylation was reached, the enzymes were tested in the menadione reductase assays. The reduction assay mixture consisted of 100 mM potassium phosphate buffer, pH 6.9, 200 mM KCl, 1 mM EDTA, and 100 μM NADPH in a total volume of 1 mL. Menadione reductase activity was determined by recording the initial velocities in the presence of increasing naphthoquinone concentrations (0−300 μM) after addition of aliquots of the prereacted enzymes. Menadione was first dissolved in DMSO, and a final 1% DMSO concentration was kept constant in the menadione reductase assays. To measure the intrinsic NADPH oxidase activity in the absence of the naphthoquinone, the reaction was started by adding GR as the last component in the cuvette containing 1% DMSO. For the determination of K_m and V_{max} values, the steady-state rates were fitted by using nonlinear regression analysis software (Kaleidagraph) to the Michaelis−Menten equation.[20] From these values, the turnover number k_{cat} and the catalytic efficiency k_{cat}/K_m were calculated. The initial rate for intrinsic NADPH oxidation activity was compared with the rates measured in the presence of the naphthoquinone for unreacted and alkylated enzymes.

Rapid-Reaction Studies with the EH_2 and EH_4 Species of *P. falciparum* Glutathione Reductase. EH_2 and EH_4 were prepared by titrating anaerobic enzyme with NADPH in a tonometer equipped with a side arm cuvette and an attached gastight syringe containing the NADPH solution. Enzyme solutions for rapid-reaction studies were made anaerobic in glass tonometers by repeated cycles of evacuation and equilibration over an atmosphere of purified argon. Reduction of 24.7 μM *P. falciparum* GR with 1 equiv of NADPH led to the thiolate−flavin charge-transfer complex $EH_2(FAD)(SH)_2$ with its characteristic absorbance at 540 nm. The preparation of the EH_4 form was performed separately by using an NADPH-regenerating system consisting of glucose-6-phosphate dehydrogenase and glucose 6-phosphate as previously described.[4] In the titration experiment, the anaerobic cuvette contained 28 μM GR (4 mL), 1 mM glucose 6-phosphate and 1 unit of glucose-6-phosphate dehydrogenase (at 25 °C), and 7.7 μM NADPH and the reaction was allowed to proceed for 3.5 h. Formation of EH_2 and EH_4 was assessed spectrophotometrically using a Hewlett-Packard 8452A diode array spectrophotometer. Stopped-flow experiments were performed on a Hi-Tech SF-61 DX2 instrument at 4 °C. Solutions of menadione for rapid reaction studies were made anaerobic by bubbling them with purified argon within the syringes that were to be loaded onto the stopped-flow instrument. Time courses were fitted to the sum of two or more exponential functions (eq 1, where A_t is the absorbance at time t, A_1 to A_3 are the amplitudes of the phases of reaction described by the observed pseudo-first-order rate constants, k_1 to k_3, and c is a constant).

$$A_t = A_1 e^{-k_1 t} + A_2 e^{-k_2 t} + A_3 e^{-k_3 t} + c \qquad (1)$$

Protein Modification and Crystallization. Recombinant human GR purified according to ref 21 had a specific activity of 182 U/mg (9500 units/μmol of subunit). For modifying the enzyme with fluoro-M_5, 36 μg/mL GR subunits were added to 100 mL of GR assay buffer, 5 μM fluoro-M_5 (7.3 equiv/enzyme subunit, 0.05% DMSO final), and 100 μM NADPH at room temperature. GR activity was measured every 15 min using the GR assay. After 90 min, inhibition of 96% was observed. Subsequently, the incubation mixture was concentrated by centrifugation through a Centriprep YM-30 filter device (Amicon) at 3000*g* to give a final volume of 300 μL. The protein was then dialyzed overnight at 4 °C against the crystallization buffer (100 mM potassium phosphate, pH 7.5, 120 mM ammonium sulfate). Crystals were grown at 15 °C using the hanging-drop technique in 24-well plates with 100 mM potassium phosphate, pH 8.0, and 640 mM ammonium sulfate as

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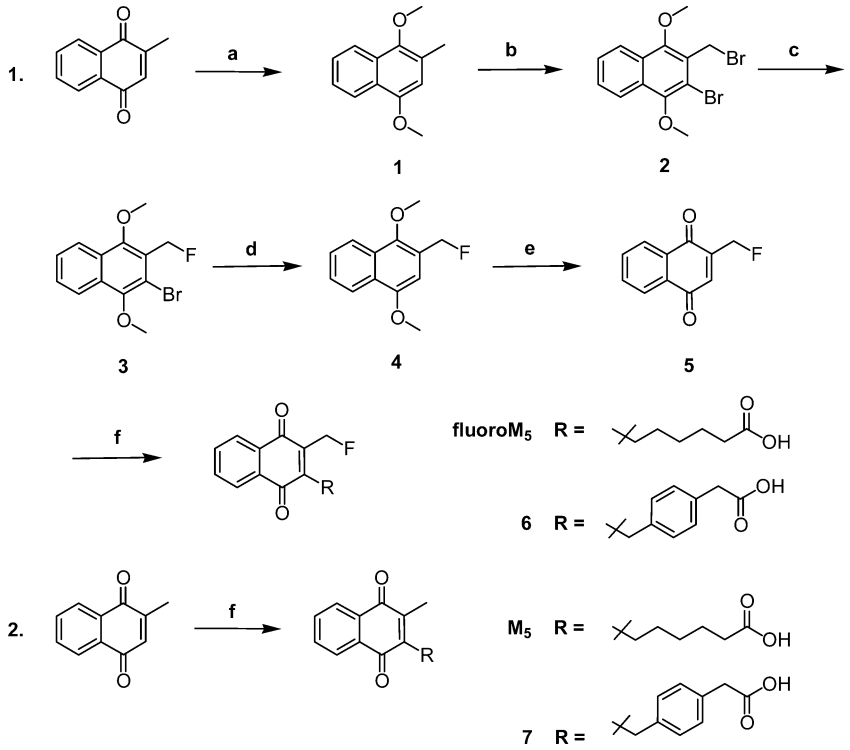
Scheme 3. Synthesis Fluoro-M_5 and Analogues[a]



[a] Conditions: (a) (1) 3.0 equiv of $SnCl_2$/HCl, EtOH, rt; (2) 3.0 equiv of $(CH_3O)_2SO_2$, 4 equiv of KOH, acetone, 60 °C; (b) (1) 1.05 equiv of NBS, CCl_4, 2 h, reflux; (2) 1.05 equiv of NBS, 0.1 equiv of BPO, 2 h, reflux; (c) 2 equiv of TBAF·$3H_2O$, CH_3CN, 2 h, rt; (d) 1.1 equiv of BuLi, 15 min, −78 °C, then quenching; (e) 3 equiv of CAN, CH_3CN/H_2O, 15 min, rt; (f) 3.0 equiv of pimelic acid or 1,4-phenylenediacetic acid, 0.1 equiv of $AgNO_3$, 1.3 equiv of $NH_4S_2O_8$, 2.5 h, 70 °C.

the reservoir buffer. In the drop, the initial protein concentration was ~10 mg/mL, and the concentration of fluoro-M_5 was 100 μM. Prior to the X-ray diffraction experiment, crystals were soaked in buffer (10 mM potassium phosphate, pH 7.5, 1.5 M ammonium sulfate) with an initial concentration of 0% glycerol and transferred in 10 steps to buffers with increasing glycerol content to give a final concentration of 20% glycerol.

Data Collection and Structure Determination. All diffraction data were recorded by the rotation method and processed by XDS, which includes routines for space group determination, scaling, and conversion to reduced structure factor amplitudes.[22] The crystals obey $P2_1$ space group symmetry and contain two monomers in the asymmetric unit. The data set (Table S1, Supporting Information) was collected at 100 K using synchrotron radiation at the ESRF (Grenoble, France, beamline ID14-4, rotation/image 1.0°, wavelength 0.93927 Å, Quantum4 CCD detector (ADSC)). Reflection phasing and structure refinements were carried out using the CNS program of Brünger et al.,[23] and model correction was done with the graphic program O.[24] The structure was solved by molecular replacement, using unmodified human glutathione reductase (1gra, 1) as the search model. The initial difference map ($|F_o| - |F_c|$) revealed the presence of electron density showing M_5 linked via a thioether bond at the active sites of the two monomers in the asymmetric unit. The model displays good stereochemistry as verified by Procheck.[25] The geometry of 6-[2′-(3′-thiomethyl)-*1′*,*4′*-naphthoquinolyl]hexanoic acid was built with GaussView and refined with molecular mechanics methods using the UFF force field (Gaussian, Inc.).

Results

Chemistry. Initial attempts to synthesize 2-(fluoromethyl)-1,4-naphthoquinone (**5**) by bromination of menadione and subsequent nucleophilic substitution by fluoride using tetrabutylammonium fluoride trihydrate (TBAF) were not successful. Neither the variation of the conditions with respect to solvent and pH nor the use of inorganic fluorides gave the desired product. This is most probably due to the basicity of fluoride that promotes elimination processes, resulting in complete degradation. We have therefore followed a route via reduced and *O*-methylated menadiol **1** (Scheme 3, entry 1). The subsequent step involves the bromination of the aromatic core and the side chain to yield 2-bromo-3-(bromomethyl)-1,4-dimethoxynaphthalene (**2**).[26] The benzylic bromine can be quantitatively substituted by fluorine by incubation with hydrated tetrabutylammonium fluoride in acetonitrile.[27] This method is straightforward and more convenient than using alkali-metal or silver fluoride.[28,29] Debromination of the fluoro intermediate **3** was performed by BuLi treatment followed by quenching with AcOH. The protonated product **4** was subsequently oxidized to the respective naphthoquinone **5** by cerium ammonium nitrate (CAN), which is a well-established oxidation reagent in naphthoquinone chemistry.[26,30] The introduction of the carboxyalkyl chain was performed using a silver(II)-mediated radical decarboxylation reaction previously reported

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Table 1. IC_{50} Values for M_5 Derivatives as Inhibitors of *P. falciparum* and Human Glutathione Reductases

	IC_{50} (μM)			IC_{50} (μM)	
compd	*P. falciparum* GR assay[a]	human GR assay[a]	compd	*P. falciparum* GR assay[a]	human GR assay[a]
menadione	42.0	27.5			
M_5	4.5[b]	3.2[b]	**6**	2.0	3.5
fluoro-M_5	6.4	4.1	**7**	7.7	1.4

[a] The values were determined at pH 6.9 and 25 °C in the presence of 1 mM GSSG. [b] Data from ref 14.

for M_5 (Scheme 3, entry 2) and applied to combinatorial synthesis of naphthoquinones.[16,31] The reaction leads to a very selective alkylation at the free position of the quinone moiety.[16,32] This reaction was applied for the preparation of the new carboxylic acids fluoro-M_5, **6**, and **7** (Scheme 3, entries 1 and 2) starting from pimelic acid and 1,4-phenylenediacetic acid and the menadione derivatives, menadione itself, and the (fluoromethyl)menadione **5**.

Electrochemical Reduction of Substituted Menadione Derivatives. The redox potentials of the non-fluoro- and the fluoronaphthoquinones were determined by cyclic voltammetry to study the influence of the fluorine atom on the $\pi-\pi$ electron acceptor properties (Table S2, Supporting Information). For comparison with menadione, the introduction of the hexanoic acid chain at the C-3 of menadione in M_5 produced a shift to more negative potentials on both waves showing one-electron- and two-electron-transfer reactions (Table S2, Supporting Information). Thus, as we previously reported,[14] M_5 is a poorer substrate of both GRs than menadione because it is less readily reduced. Two interesting conclusions can be drawn from the electrochemical behavior of the new compounds. First the substitution of the benzyl chain at C-3 of menadione leads to a more positive $E_1°$ value (-0.69 V) for **7** compared to the $E_1°$ value (-0.75 V) for M_5. These values might suggest stronger electron-donating properties of the hexane chain at C-3 in M_5 versus the benzyl chain in **7**. The presence of one fluorine atom at the methyl side chain of both menadione derivatives fluoro-M_5 and **6** shifts both one-electron- and two-electron-reduction waves to more positive potentials, rendering these (fluoromethyl)menadione derivatives more reducible than the non-fluoro analogues. This behavior was favorable for the design of fluoro-based suicide substrates for both GRs. The combined effects due to the presence of both the fluorine atom at the methyl position and the benzyl chain at the C-3 of the menadione core are additive in the resulting GR inhibition, as indicated by the low IC_{50} values determined for **6** versus fluoro-M_5 in both GR assays and versus M_5 in *P. falciparum* GR assays (Table 1).

Inhibition of Human Glutathione Reductase under Steady-State Conditions. Determination of the IC_{50} values of the compounds fluoro-M_5, **6**, and **7** in comparison with M_5 under steady-state conditions was performed in the assay using 1 mM GSSG. As previously discussed, this high GSSG concentration does not mimic physiological conditions but cell-pathological conditions where the GSSG level starts to be toxic for the parasite. Very few inhibitors described earlier in the literature were found to be potent inhibitors in GR assays: methylene blue, some arylisoalloxazines, and M_5[14] displayed IC_{50} values ≤ 10 μM. Under these specific conditions M_5 is the most potent known inhibitor of both *P. falciparum* GR and human GR, displaying IC_{50} values of 4.5 and 3.2 μM, respectively.[14] The bioisosteric replacement of three methylene groups by a phenyl group in **7** improved the inhibitory potency of the parent M_5 with respect to the human enzyme and in the fluoro analogue **6** with respect to the *Plasmodium* enzyme (Table 1).

To investigate the inhibition of human GR at varying GSSG concentrations (40−1000 μM), kinetic studies were first performed with saturating NADPH concentration (100 μM) in the presence of fluoro-M_5 (0−10 μM). A K_i value of 5.4 ± 0.4 μM for fluoro-M_5 was determined as shown in panel C, Figure S1 (Supporting Information). The Cornish-Bowden plot (panel A, Figure S1) as well as the Lineweaver−Burk plot (panel B, Figure S1) for fluoro-M_5 was consistent with noncompetitive inhibition, whereas equations for competitive and uncompetitive inhibition could not accurately describe the observed relationship between v and GSSG concentration.

Glutathione Reductase-Catalyzed Naphthoquinone Reductase Activity. The ability of *P. falciparum* GR to reduce the naphthoquinone moiety was studied by following the oxidation of NADPH in the presence of **7**. The naphthoquinone reductase activity catalyzed by *P. falciparum* GR was compared to the intrinsic NADPH oxidation activity of the enzyme in the absence of naphthoquinone (NQ) (eq 2). *P. falciparum* GR displayed a 7.4-fold higher NADPH oxidation activity in the presence of 200 μM **7** (eq 3 or 4),

$$2O_2 + NADPH + H^+ \rightarrow 2O_2^{\bullet -} + NADP^+ + 2H^+ \quad (2)$$

$$2NQ + NADPH + H^+ \rightarrow 2NQ^{\bullet -} + NADP^+ + 2H^+ \quad (3)$$

$$NQ + NADPH + H^+ \rightarrow NQH_2 + NADP^+ \quad (4)$$

the NADPH oxidation rates being 0.012 U/mg of protein in the absence of naphthoquinone and 0.089 U/mg of protein in the presence of 200 μM **7**. The K_m and k_{cat} values, 115 ± 23 μM and 0.24 ± 0.02 s^{-1} corresponding to $V_{max} = 0.25$ U/mg of protein, were derived from measurements at six different substrate concentrations. When NADPH consumption was followed, **7** was reduced by *P. falciparum* GR with a catalytic efficiency k_{cat}/K_m of 2.1×10^3 M^{-1} s^{-1}, a value 1.1-fold and 2.3-fold higher than the values determined for menadione and M_5,[14] respectively. When **7** was studied as a substrate of human GR, the K_m and k_{cat} values were determined as 355 ± 91 μM and 0.34 ± 0.06 s^{-1}, respectively. This results in a catalytic competence k_{cat}/K_m of 0.9×10^3 M^{-1} s^{-1}, a value 5.6-fold lower than that determined for menadione. For comparison, M_5 when measured up to 200 μM was found not to be a substrate of the human GR.

Reaction of Fluoro-M_5 with Glutathione Reductases from Man and *P. falciparum*. The binding locus of the lead structure M_5 has remained unknown. On the basis of the kinetic data, it is clear that the parent compound M_5—from which our new mechanism-based inhibitor fluoro-M_5 derives—is an uncompetitive inhibitor with respect to both substrates of glutathione reductase, NADPH and GSSG.[14] This was found to be true for both glutathione reductases from man and from *P. falciparum*. Since cocrystallization of M_5 and M_5-derived reversible inhibitors with the respective enzymes was not successful, we followed the approach of labeling the binding site by an

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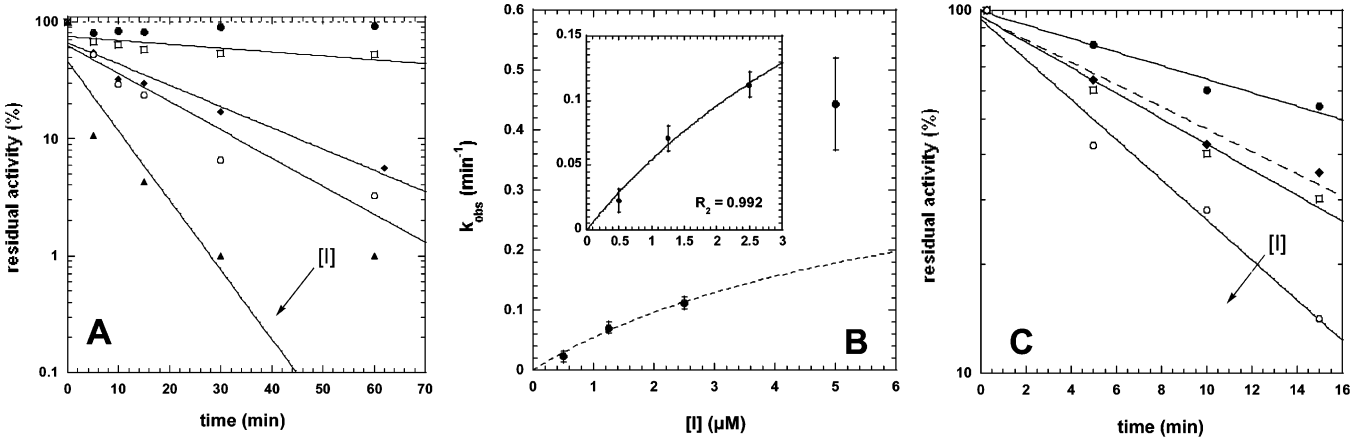


Figure 1. Time-dependent inactivation of human and *P. falciparum* glutathione reductases by fluoro-M_5. (A) Human GR (6.85 pmol in 50 μL) was incubated in the presence of 160 μM NADPH and inhibitor for 0, 5, 10, 15, 30, and 60 min incubation periods. Fluoro-M_5 concentrations used were 0 (●), 0.5 (□), 1.25 (◆), 2.5 (○), and 5.0 (▲) μM. A 2% final concentration of DMSO was present in all incubation mixtures. (B) The k_{obs} data versus [I] were fitted to eq 5 (see the text), which resulted in the hyperbolic curve (dashed line and zoom in the inset). At low inhibitor concentration the dissociation constant for fluoro-M_5 and the first-order rate constant for irreversible inactivation were determined as $K_I = 7 \pm 5$ μM and $k_i = 0.4 \pm 0.2$ min^{-1}, respectively. (C) The time dependency of inactivation of *P. falciparum* GR was revealed by determining the residual activity of different aliquots of incubated enzyme (21 pmol in 50 μL) for different time periods: 0.25, 5, 10, and 15 min. Fluoro-M_5 concentrations used were 0 (●), 10 (□), 25 (◆), and 50 (○) μM. A 2% concentration of DMSO was present in all cases.

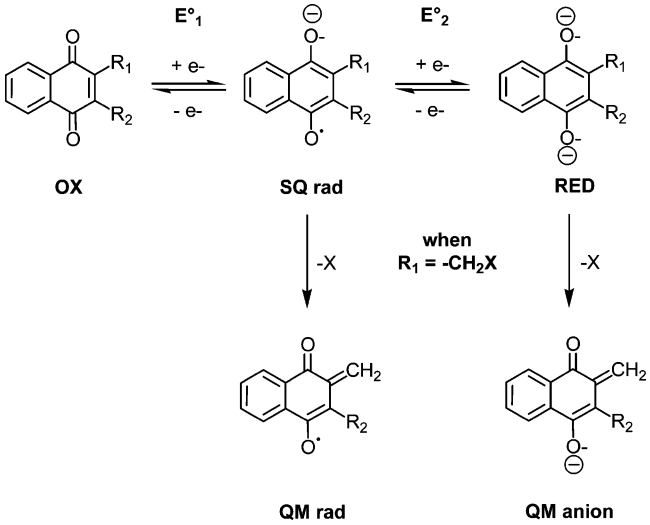
irreversible mechanism-based inhibitor closely related to M_5 in structure. This lead compound is a weak substrate of glutathione reductase from *P. falciparum*, the k_{cat} and K_m values being 0.10 s^{-1} and 109 μM.[14] The specific activity as low as 0.11 U/mg for *P. falciparum* GR-catalyzed reduction of M_5 does not interfere with our approach to a mechanism-based inhibitor that is activated in a reduction process directly catalyzed by the GR enzyme (Scheme 2). Also the presence of one fluorine atom may have an influence on the oxidant character of the parent core M_5. The electrochemical properties of the newly synthesized compounds were also evaluated in parallel.

Inactivation of human GR by fluoro-M_5 followed pseudo-first-order reaction kinetics. The experimental data allowed application of the derivation by Kitz and Wilson[33] for irreversible inactivation. A semilogarithmic plot (panel A, Figure 1) of the fraction of noninhibited enzyme activity $\ln(v_i/v_0)$ versus incubation time yielded linear curves with increasing slopes, equivalent to the apparent rate constant of irreversible inhibition (k_{obs}). The k_{obs} values for fluoro-M_5 and human enzyme ranged between 22.7×10^{-3} and 443×10^{-3} min^{-1} within the log-linear range of the inhibition curve (panel B, Figure 1). The secondary plot expressing k_{obs} as a function of inhibitor concentration followed eq 5, where K_I represents the dissociation

$$k_{obs} = \frac{k_i[I]}{K_I + [I]} \qquad (5)$$

constant of the inhibitor and k_i is the first-order rate constant for irreversible inactivation. It was illustrated for fluoro-M_5 and the human GR, by a hyperbolic curve only at low inhibitor concentration, allowing estimation of k_i as 0.4 ± 0.2 min^{-1}, $K_I = 7 \pm 5$ μM, and second-order rate constant k_i/K_I as 1×10^3 M^{-1} s^{-1} (inset, panel B, Figure 1). The resulting half-time value ($t_{1/2}$) of inactivation of the human GR was determined as 1.7 min. A double-reciprocal replot of k_{obs} versus [I] fitted to a linear relationship (data not shown). At higher inhibitor concentration, the curve was not hyperbolic anymore but became polynomial,

Scheme 4. Oxidoreduction of Naphthoquinones[a]



Abbreviations: OX, oxidized form; RED, reduced form; SQ rad, semiquinone radical; QM rad, quinone methide radical; QM, quinone methide.

suggesting that more than one binding site of the naphthoquinone is involved in the presence of different enzyme species of reacted and unreacted enzymes in mixture (Scheme 5). Secondary binding sites with lower affinity are well-known for the related enzyme trypanothione reductase.[34] Similar to the recent observation for a nitrofuran compound and the human thioredoxin reductase,[35] increased naphthoquinone reductase and NADPH oxidase activities of enzyme alkylated by fluoro-M_5 obscure the inactivation of the unreacted enzyme, rendering the measurement of the residual activity more complex (see the highest inhibitor concentration and the longest time in panel A of Figure 1). The results are consistent with our concept of mechanism-based inhibition by (monofluoromethyl)menadione derivatives. In additional kinetic experiments (panel C, Figure

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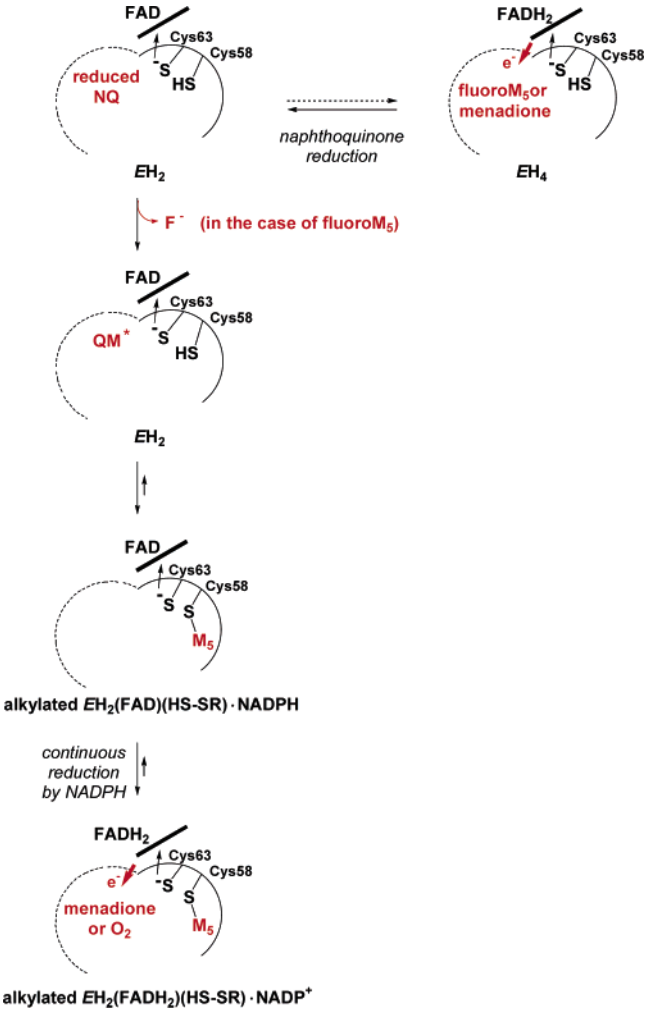
Scheme 5. Sketch of the Postulated Alkylation Mechanism of GR by Fluoro-M_5 and of Oxidation of Reduced GR by Menadione[a]



[a] The dashed arrow means that EH_4 was formed in an independent experiment using the NADPH-regenerating system and might be generated from EH_2 or another enzyme intermediate described in Scheme 1. Naphthoquinone (fluoro-M_5 or menadione) reduction by EH_4 generates EH_2. Upon reduction by the EH_4 species, the reduced naphthoquinone (NQ) undergoes fluorine elimination. The released highly reactive quinone methide (QM*) is attacked by the solvent-exposed thiol Cys58, in accordance with the crystal structure of the human alkylated GR active site. After alkylation of Cys58, NADPH oxidase and naphthoquinone reductase activities can be continuously maintained at the expense of NADPH.

1), we observed that *Plasmodium* GR was also quickly inactivated upon incubation of the enzymes with NADPH and fluoro-M_5. The first-order rate constant k_i for irreversible inactivation could not be determined because of the concomitant inactivation of the parasitic enzyme by NADPH itself.[36] In addition, the resulting parasitic GR inhibition is very complex because the irreversible component resulting from incubation of enzyme, NADPH, and fluoro-M_5, is partly masked by both the redox activity of the free compound and the increased NADPH oxidase activity of the alkylated enzyme. This last activity was also reported for the related *P. falciparum* thioredoxin reductase inactivated by 6,7-dinitroquinoxaline[37] and the human thioredoxin reductase alkylated by chlorodinitrobenzene.[38] Although covalent inactivation of both enzymes was clearly observed, the kinetics of inactivation indicate that fluoro-M_5 interacts in a more complex way with *P. falciparum* GR than with human GR (data not shown).

Dialysis of the alkylated enzyme solution and subsequent enzymatic assays clearly showed that the type of inhibition was indeed irreversible since GSSG reduction was completely suppressed. Incubation of the enzymes with inhibitor in the absence of NADPH did not result in any inactivation, indicating that—as predicted from our model—reduction of the enzyme is a prerequisite for this mechanism-based inactivation.

Naphthoquinone Reductase Activity of Human and *P. falciparum* Alkylated Glutathione Reductases. The ability of human and *P. falciparum* fluoro-M_5-alkylated GRs to reduce menadione was studied by following NADPH oxidation. Complete alkylation for both enzymes was revealed by full inactivation of the glutathione reductase activity as described in the Experimental Section. The naphthoquinone reductase activity (eqs 3 and 4) of both alkylated GRs was first compared to the intrinsic NADPH oxidase activities in the absence of naphthoquinone (eq 2). Human and *P. falciparum* alkylated GRs displayed a 3-fold higher NADPH oxidase activity in the presence of oxygen (eq 2) than unreacted enzymes; the observed NADPH oxidation rates in the absence of menadione were 0.036 U/mg for human alkylated GR and 0.031 U/mg for parasitic alkylated GR. Then the menadione reductase activities of human and *P. falciparum* alkylated GRs were compared to those of the unalkylated enzymes. Human and *P. falciparum* alkylated GRs showed increased rates in the reduction of menadione (Figure 2) compared to their unmodified forms (k_{cat} of 0.16 s^{-1} for both unreacted GRs).[14] The k_{cat}/K_m values for human and *P. falciparum* alkylated GRs, 1.23 and 3.55 mM^{-1} s^{-1} (corresponding to V_{max} = 0.41 U/mg human GR and 0.81 U/mg parasitic GR) were derived from measurements at five different substrate concentrations. For the unmodified enzymes from man and *P.* falciparum k_{cat}/K_m values of 1.50 and 1.94 mM^{-1} s^{-1} were determined under the same conditions.

Comparison of the Oxidation of EH_2 and EH_4 Forms of *P. falciparum* Glutathione Reductase by Menadione. We generated the EH_2 and EH_4 forms of *P. falciparum* glutathione reductase and measured the rates of oxidation of these two enzyme forms by menadione in anaerobic stopped-flow experiments. The two-electron-reduced enzyme [$(EH_2)(FAD)(SH)_2$] has thiolate−flavin charge-transfer absorbance at 540 nm, while the four-electron-reduced enzyme (EH_4), where both the cysteines and the flavin are reduced, has no absorbance from the charge-transfer complex or oxidized flavin. The reactions of EH_2 and EH_4 with menadione were studied in anaerobic stopped-flow experiments (Figure 2B,C). EH_2 reacted very slowly with 150 μM menadione. After a long lag, oxidized enzyme, monitored by the increase in absorbance at 485 nm, appeared after several hundred seconds (Figure 2B). This corresponded to the disappearance of charge-transfer absorbance at 540 nm. Fitting the 485 nm trace to sums of exponentials (eq 1) gave an apparent rate constant for the formation of E_{ox} of 0.0089 s^{-1} (a half-life of 77 s); the same value was obtained

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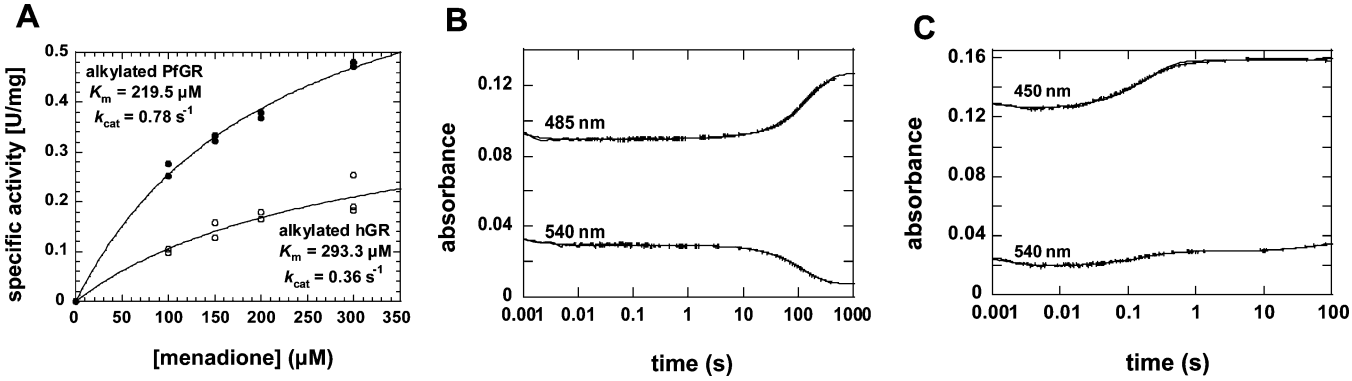

Figure 2. Menadione reductase activity of alkylated glutathione reductases and of EH_2 and EH_4 forms of *P. falciparum* glutathione reductase. (A) The menadione reductase activity of human (○) and *P. falciparum* (●) GR alkylated by fluoro-M_5 was tested by monitoring the oxidation of NADPH at 340 nm, 25 °C, and pH 6.9 under steady-state conditions. Menadione was dissolved in DMSO, and the NADPH oxidation activity was measured at five different concentrations in duplicate (0−300 μM) in the presence of 1% DMSO. For the determination of K_m and V_{max} values, the steady-state rates were fitted by using nonlinear regression analysis software (Kaleidagraph) to the Michaelis−Menten equation,[20] and the turnover number k_{cat} and the catalytic efficiency k_{cat}/K_m were calculated. The initial rate for aerobic NADPH oxidation activity of modified *P. falciparum* GR was not subtracted from the rates measured in the presence of the naphthoquinone because it proved negligible in comparison to that of GR-catalyzed menadione reductase activity. (B, C) Stopped-flow absorbance traces after mixing 24.7 μM *P. falciparum* EH_2 (B) or 28 μM *P. falciparum* EH_4 (C) with 150 μM menadione at 25 °C and pH 6.9 and monitored at the indicated wavelengths. The bold points are the experimental data, and the solid lines represent data fitted according to eq 1. Note the logarithmic time scales.

for the disappearance of charge-transfer absorbance at 540 nm. Interestingly, EH_4 reacted much faster with menadione, forming EH_2 within ~1 s. After this prominent initial phase, smaller, slower phases occurred in the 100 s reactions. Traces were fitted to sums of exponentials, giving an apparent rate constant for the formation of EH_2 from EH_4 of 6.0 s^{-1} (a half-life of 0.12 s) at both 450 and 540 nm (Figure 2C). The other phases, which might be due to traces of EH_2 in the EH_4 preparation, were much slower and were not considered catalytically significant (data not shown). Thus, reduction of 150 μM menadione by EH_4 was about 660-fold faster than by EH_2, suggesting that EH_4 is the species primarily responsible for menadione reduction. In contrast, the reduction of GSSG occurs exclusively by EH_2.

Crystal Structure of Human Glutathione Reductase Inactivated by Fluoro-M_5. For crystallographic analysis of the alkylated enzyme we selected the human enzyme because it crystallizes readily even in the presence of inhibitors. The 3D structures of unliganded human GR and of various ligand−protein complexes are known.[39,40] Also, it is a good model for the parasitic enzyme since the crystal structure of unliganded *P. falciparum* GR has been solved[39] and shows a scaffold very similar to that of human GR.

We obtained monoclinic crystals (space group $P2_1$, Table S1, Supporting Information) of human GR, alkylated by the postulated quinone methide generated from fluoro-M_5. The refined structure revealed the biological dimer of human GR in the asymmetric unit of the crystal. The two catalytic sites are occupied by M_5 linked via a thioether bound. Except for the first 17 N-terminal residues and the side chains of Phe78 and Phe78′, the structure is well represented by the electron density. The side chains of nine residues in the asymmetric unit assume alternative conformations. Our structure was refined at 1.7 Å resolution with an R_{free} of 25.6% and with an overall temperature factor of 30.3 Å^2 (Table S1, Supporting Information). All backbone torsion angles are within the allowed region of the Ramachandran plot. The structural data indicate a clear stoichiometry with one inhibitor molecule bound per enzyme subunit of the homodimeric GR. The binding site of the inhibitor is characterized by covalent and noncovalent interactions. The compound is covalently linked to Cys58 of the active site via a sulfur−carbon bond at the position previously occupied by the fluorine atom (Figure 3). This is in full agreement with the prediction that a nucleophilic residue in the enzyme should easily add to the highly reactive quinone methide generated as the inhibitor intermediate formed after reduction and elimination of the leaving fluorine atom (Scheme 2). The inhibitor is covalently bound, but it interacts with the enzyme by additional

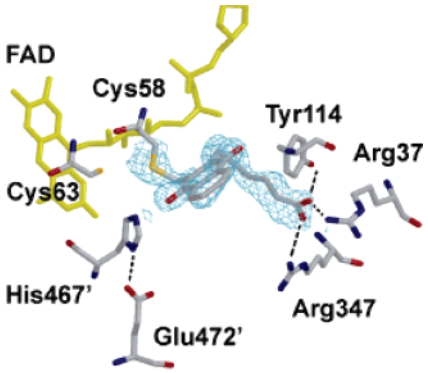

Figure 3. Close-up of the human GR active site showing the alkylation of Cys58 by the naphthoquinone methide. The superimposed difference electron density omit map is contoured at 3σ. Relevant residues in the surroundings of the inhibitor are shown. The FAD molecule is drawn with lemon-yellow sticks. Inhibitor and residues are depicted with gray (C atoms), blue (N atoms), red (O atoms), and gold-yellow (Sγ atoms) sticks. The structure, solved at 1.7 Å resolution, supports our proposed mechanism: after reduction of fluoro-M_5 the reactive intermediate undergoes fluorine elimination to yield the quinone methide, which is a highly reactive Michael acceptor. The nucleophile is the most solvent exposed cysteine (Cys58) of the catalytic site, which is also the residue responsible for the thiol−disulfide exchange with the substrate GSSG. The crystal structure shows a stoichiometry of one covalently bound inhibitor molecule per enzyme subunit. Furthermore, defined noncovalent interactions were observed. The carboxylate function of the M_5 moiety is in ionic interaction with the two active site residues Arg37 and Arg347 and is stabilized by a hydrogen bond with Tyr114. The figure was drawn with bobscript and Raster3D.[50−52]

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noncovalent interactions. One keto group of the naphthoquinone is bound via a hydrogen bridge to His467′ of the other subunit. The conformation of the histidine is further stabilized by the interaction with Glu472′ (Figure 3). The carboxylate function of the inhibitor side chain is in ionic contact with the active site residues Arg37 and Arg347 and is hydrogen-bonded to Tyr114. Arg37 is known to be important for binding the negatively charged glycine carboxylate in natural substrate glutathione disulfide.[41] This finding is consistent with our previous finding that the carboxylate function of M_5 seems to be essential for targeting the inhibitor to the active site. On the basis of the structural information, we can further conclude that the binding site of the inhibitor is specific and well defined because no further electron densities could be detected at other sites.

Antiparasitic and Cytotoxic Activities in Vitro. Inhibition of the growth of *P. falciparum* by our naphthoquinones was evaluated by determining the inhibitor concentration required to kill 50% of the parasite (ED_{50} values). The antimalarial activities of M_5 and its derivatives against the intraerythrocytic *P. falciparum* strains 3D7 and K1 are given in Table S3 (Supporting Information). The cytotoxicity of the compounds for human cells was determined in assays using a human nasopharyngeal carcinoma (KB) cell line to enable an estimation of the therapeutic index. This parameter is defined by the ratio of the ED_{50} value measuring antimalarial efficacy over the ED_{50} value measuring toxicity. Interestingly, the weaker cytotoxic effects against human cells are caused by the reversible GR inhibitors M_5 and **7** while the more pronounced toxic effects are due to the irreversible enzyme inhibitors fluoro-M_5 and **6** and to the nonspecific inhibitor menadione. In particular, compound **7** showed the highest ED_{50} value against the human KB cell line, giving a therapeutic index of ca. 47−52 with respect to K1 and 3D7 strains, respectively. In addition compound **7** is as effective against the chloroquine-sensitive strain 3D7 as against the chloroquine-resistant strain K1. The therapeutic index of menadione is low, ranging from 2.8 to 3.5, probably because the compound is known to bind to different targets. The most potent and irreversible inhibitor **6** displayed higher cytotoxicity against both human cells and parasites, resulting in low therapeutic indexes of 1.6−0.4 for 3D7 and K1 strains, respectively. On the basis of these observations, the activity profile of the newly prepared naphthoquinones might be selectively exploited to design antimalarial drug candidates by optimizing the reversible and specific GR-catalyzed redox cyclers. In contrast, the irreversible GR inhibitors are more appropriate as antitumoral agents, as illustrated with the severe generalized GR deficiency after antitumor chemotherapy with carmustine, also called BCNU (1,3-bis(chloroethyl)-1-nitrosourea).[42]

Discussion

Design and Chemistry. 2-(Fluoromethyl)menadione derivatives were first designed as suicide substrates of vitamin K epoxide reductase—which is not a flavoenzyme—but turned out *not* to be time-dependent inhibitors.[28] In addition, the attempted synthesis of 2-fluoromethyl vitamin K and of its 2,3-epoxide led to decomposition of the naphthoquinone in enzyme assays, and the chemical preparation yielded only 2% of the desired epoxide.[28] In our hands, the reported route[28] based on the coupling of a lithium dialkyl cuprate with a bromide failed. Instead a new route (Scheme 3) was successfully achieved by improvement of two key steps: (i) Finkelstein exchange of bromine by fluorine using tetrabutylammonium fluoride, (ii) alkylation of 2-(fluoromethyl)menadione using the radical decarboxylation of carboxylic acids by silver nitrate and ammonium peroxodisulfate. The developed route proved to be convenient for synthesizing a large array of diversely 3-substituted 2-(fluoromethyl)menadione derivatives, as illustrated by the synthesis of fluoro-M_5 and its analogue **6**. Furthermore, the rationale given for the GR inhibition mechanism appears to be valid since inactivation of the enzyme occurred at a high rate and led to the alkylated protein as hypothesized in Scheme 2 and proven by the crystal structure of human alkylated GR. In particular, the parasitic alkylated enzyme, inactivated in its physiological antioxidant function, retained increased NADPH oxidase activity that might contribute to high oxidative stress in the parasites along with GSSG accumulation and continuous waste of the NADPH level.

Kinetic Studies and Electrochemistry. Human GR was identified as an attractive target for the development of new antimalarials and antitumor molecules,[5] in particular to circumvent drug-resistance mechanisms. Among all types of inhibitors, the turncoat inhibitors—also called subversive substrates or redox cyclers—and the irreversible inhibitors are the most promising ones. As previously shown, most of the subversive substrates, including menadione itself and its derivative M_5, displayed noncompetitive or uncompetitive inhibition with respect to both NADPH and GSSG.[6,43,44] Especially the uncompetitive type of inhibition makes these lead compounds so interesting for a potential drug use because this inhibitor type cannot be overcome by physiological counteractions such as increase of the substrate concentration or increase of the enzyme expression.[45] Uncompetitive inhibitors only bind to enzyme−substrate complexes, implying that any cellular increase in substrate or enzyme concentration even reinforces inhibition. This can lead to dramatic cell responses because of the toxic accumulation of the substrate.[45] The identification of the binding sites of subversive substrates—such as naphthoquinones, methylene blue, and nitrofuran derivatives—is still the most challenging issue in drug discovery applied to disulfide reductases. In agreement with kinetic data obtained with various un/noncompetitive naphthoquinones, the crystallographic structures of ligand−human GR complexes revealed that many ligands, including menadione,[46] 3,7-diamino-2,8-dimethyl-5-phenylphenazinium chloride (safranin),[46] 6-hydroxy-3-oxo-3*H*-xanthene-9-propionic acid,[43] a series of 10-arylisoalloxazines,[44] *S*-(2,4-dinitrophenyl)-glutathione,[3] and methylene blue,[39] are bound in a large cavity at the dimer interface. The cavity at the interface of the homodimer in human GR is linked to the GSSG binding site via two primary channels.[16] For instance, menadione was shown to bind sandwiched by the Phe78/Phe78′ pair in the cavity at the interface of the homodimer.

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In this study, new M_5 analogues were designed and synthesized for optimizing GR inhibition. The bioisosteric replacement of three methylene groups by a phenyl ring in compound **7** has a direct effect on the redox behavior of the menadione moiety by affecting both one-electron- and two-electron-reduction rates in agreement with their $E_1°$ and $E_2°$ values (Table S2, Supporting Information). However, the redox behavior is not the single criterion that influences the inhibitory capability of subversive substrates since menadione and **7** possess similar oxidant properties, i.e., similar $E_1°$ and $E_2°$ values, but they displayed very different IC_{50} values in both GR assays (Table 1). This suggests that inhibition depends on both the redox potentials and on structural features governing recognition by the enzyme. To get a deeper insight into the binding locus of the inhibitors, we have designed and synthesized irreversible inhibitors based on M_5 and **7**. Introduction of one fluorine atom in a β position to the carbonyl group of the menadione core was aimed at the conversion of the reversible inhibitors M_5 and **7** into mechanism-based inhibitors or suicide substrates, fluoro-M_5 and **6**, to inactivate both enzymes. First, fluorine significantly affected both one-electron- and two-electron-reduction potentials of the fluoro-M_5 analogues, i.e., $E_1°$ and $E_2°$ values (Table S2, Supporting Information). These effects were desired to efficiently inactivate the enzyme upon reduction. The combined effects of both the fluorine and the benzyl chain in **6** lead to the lowest oxidation potentials upon formation of the semiquinonic radical and the dianionic form. The formation of one of these two reactive forms can be proposed as the first step in the mechanism of enzyme inactivation, in good agreement with the IC_{50} values in both GR assays. The choice of a fluorine atom as a leaving group was also governed by criteria such as size, stability, specificity, lipophilicity, and bioavailability. Irreversible inactivation of both GRs by both fluoro-M_5 analogues occurred at a high rate in accordance with the $t_{1/2}$ and k_i values determined for GR inactivation. As predicted, the elimination of fluorine facilitates Michael addition of a nucleophile to the quinone methide (Scheme 2), in accordance with the crystal structure of the human alkylated GR active site. The presence of a nucleophilic residue (Cys) near the region where the naphthoquinone is reduced agrees with our structural data. The highly reactive quinone methide (Scheme 2) can in principle be attacked by *N*-, *O*-, or *S*-nucleophiles.[18] It was not surprising to observe that the nascent thiol Cys58, which is the most reactive and the most solvent-exposed group, was found to be alkylated. However, inconsistencies between the structural and kinetic data—the binding at the GSSG site and the uncompetitive inhibition type, respectively—are most likely due to the presence of both EH_2 and EH_4 species in mixture and to the reaction of the generated quinone methide in the disulfide active site of EH_2 where complete alkylation of the most exposed Cys58 occurred (Scheme 5).

Besides the locus where naphthoquinone binding and reduction take place, the identification of the primary inhibitor−enzyme−substrate complex formed in the catalytic cycle (Scheme 1) represents a major aim for any inhibitor development. Our kinetic and structural data with the irreversible and mechanism-based inhibitor fluoro-M_5 unambiguously showed that reduced Cys58 from the EH_2 species, which reacts directly with GSSG, is not involved in naphthoquinone reduction because the alkylated enzyme can still reduce menadione. It is noteworthy to mention also that IC_{50} values of M_5, fluoro-M_5, and compounds **6** and **7** of Table 1 are low but not very different. This is due to the fact that reduction of naphthoquinones is thought to occur at the reduced flavin of EH_4 and not at the two-electron-reduced species where either the flavin or the redox-active Cys pair is reduced. Thus, naphthoquinone reduction and alkylation of Cys58 do not take place on the same time scale, in agreement with both the similar IC_{50} values of M_5, fluoro-M_5, and compounds **6** and **7** in Table 1 and half-life values of EH_2 (77 s) and EH_4 (~0.12 s) species determined in stopped-flow kinetics of menadione reduction. These conclusions are mainly based on detailed kinetic studies of the catalytic mechanism of the diaphorase reactions by *Mycobacterium tuberculosis* lipoamide dehydrogenase, a flavoenzyme closely related to GR.[47] Both naphthoquinone and oxygen reductions were shown to take place at the EH_4 level and not at the (EH_2)-$(FADH_2)(S-S)\cdot NADP^+$ complex or EH_2.[47] Reduction by EH_4 is likely to be responsible for noncompetitive or uncompetitive inhibition behavior published for a large panel of structurally diverse GR inhibitors. Our EH_4 experiment was done with *P. falciparum* GR because this reduced enzyme species was quantitatively produced under O_2-free conditions using an NADPH-regenerating system while the human EH_4 form was never observed. We can assume that EH_4 of human GR does form but is not visible because of the faster formation of EH_2, which is under kinetic control in the catalytic cycle. The NADPH-regenerating system supplies the electrons that drive the otherwise thermodynamically unfavored formation of EH_4. Thus, our stopped-flow kinetic data suggest that the EH_4 form of *P. falciparum* GR, although thermodynamically unfavored in the physiological catalytic cycle (Scheme 1), is the enzyme species responsible for menadione reduction (Scheme 5).

X-ray Diffraction Analysis of Fluoro-M_5-Modified Human Glutathione Reductase. Crystals of human GR, reacted with fluoro-M_5 and dialyzed, were grown as described in the Experimental Section. The difference Fourier maps of the crystallized alkylated enzyme showed electron density exclusively at the GSSG binding site with a stoichiometry of one inhibitor molecule per subunit. In the crystal structure of human GR with bound fluoro-M_5 presented here, the inhibitor is clearly located in the active site, covalently linked to the catalytic Cys58, which is responsible for the cleavage of the disulfide in the physiological substrate GSSG. However, both alkylated enzyme species are still able to reduce menadione with catalytic efficiencies as high as those previously described for unreacted enzymes. An interesting observation was that the electron density in the region of Phe78 and Phe78′ was not well defined. As recently analyzed by Wang et al.,[48] small-molecule binding can induce subtle changes in certain side chain atoms, playing an important role in ligand−protein binding. High flexibility of the side chains was revealed by increased *B* factors (>40 Å^2), most of which are located at interfaces either to the FAD or to the other subunit.[49] High disorder had also been shown to be associated with the first 17 residues and with Phe78 and

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Phe78′ in the 1.54 Å crystal structure of unliganded human GR.[49] This is also the case for human GR upon binding of fluoro-M_5. It is noteworthy that these Phe78/Phe78′ residues took a new, very defined and rigid orientation with *B* factors dropping to near 15 Å^2 upon binding of menadione[46] and 6-hydroxy-3-oxo-3*H*-xanthene-9-propionic acid.[43] However, just because menadione was found bound in the cavity at the 2-fold axis does not justify that naphthoquinone reduction occurs at that site. The electron-transfer mechanisms and pathways operating between the flavin and naphthoquinone remain speculative until structural studies identify the residues involved in the enzyme−naphthoquinone complex. On the basis of these observations, it cannot be excluded that the perturbation at Phe78/Phe78′ represents a structural change induced by the transient binding of fluoro-M_5. This increase in local protein flexibility might be significant for protein function and may therefore have important implications for structure-based design of small-molecule ligands; i.e., the possibility of subtle conformational changes at side chains of ternary complexes upon binding of pyridine nucleotide and naphthoquinone is strongly suggested. Because the cavity is rather close to the GSSG binding site, it is tempting to suggest that effector binding in this cavity might play a role in enzyme regulation in vivo by affecting the redox properties of the flavoenzyme via the α-helix 63−80.[49] Thus, the local high flexibility observed in the region of Phe78 and Phe78′ suggests a future direction of research since physiological ligands binding in the cavity at the 2-fold axis of the homodimer are still unknown.

Antiparasitic and Cytotoxic Activities in Vitro. The M_5 analogues showed activity against the malarial parasites with ED_{50} values below 7 μM for compound **7** against both chloroquine-sensitive 3D7 and chloroquine-resistant K1 strains (Table S3, Supporting Information). Introduction of fluorine at the methyl group of menadione led to the new compounds fluoro-M_5 and **6** with no increased antimalarial activities but with an improved cytotoxicity profile against tumor cells. This observation suggests another partition coefficient for the fluoro compounds and an irreversible mechanism occurring very rapidly in the human cells before reaching the parasitic compartments. The (fluoromethyl)naphthoquinones based on menadione reported here, e.g., compound **6**, may be considered in light of alternative leads for adjuvants in cancer chemotherapy.

Above all, the improvement of menadione led to the carboxylic acid **7** retaining an antimalarial activity similar to that of menadione itself against the chloroquine-sensitive 3D7 and chloroquine-resistant K1 strains, but showing much lower cytotoxicity against human cells. This suggests that **7**, the most potent M_5 analogue, may serve as a lead for further antimalarial development. Naphthoquinones have been studied in malaria mostly as starting blocks to derivatize chloroquine and related 4-aminoquinolines as drugs for use against chloroquine-resistant parasite strains.[13] However, apart from their effects as chloroquine-resistance reversers, they have also been reported to have intrinsic antimalarial activity, which in some cases has been attributed to effects on both the redox homeostasis and glutathione level.[6] Still another conclusion can be drawn from the data presented here: the GR-catalyzed redox-cycling activity and not the irreversible GR inactivation is the dominant principle to obtain more powerful antimalarial effects. Our previous double prodrugs of M_5—designed to target two essential pathways in the parasite, i.e., haem polymerization and redox equilibrium—were revealed to be active in vitro in the low nanomolar range and in *Plasmodium berghei*-infected mice.[13] Work on a new series of antimalarial double drugs based on M_5 and **7** with remarkable antimalarial activities both in vitro and in vivo is now in progress.

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Supporting Information Available: Detailed experimental procedures, spectrosopic data, and elemental analyses for the preparation and the characterization of the new compounds **4**−**7** and fluoro-M_5, data collection and structure refinement tables, protocols and results on cyclic voltammetry, and protocols and data for measuring enzyme kinetics, antimalarial activities, and cytotoxicity. This material is available free of charge via the Internet at http://pubs.acs.org.

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