Parasitol Res (2005) 97: 521–526
DOI 10.1007/s00436-005-1478-4

ORIGINAL PAPER

Kavita Arora · Arvind K. Srivastava

Antimalarial efficacy of methylene blue and menadione and their effect on glutathione metabolism of *Plasmodium yoelii*-infected albino mice

Received: 21 April 2005 / Accepted: 25 July 2005 / Published online: 11 October 2005

Abstract *Plasmodium yoelii* infection caused significant decline in the hepatic and splenic glutathione content and the activities of the key enzymes, that is, glutamate cysteine ligase (EC 6.3.2.2) and glutathione reductase (EC 1.8.1.7) of their murine host, that is, Swiss albino mice. Methylene blue as well as menadione were found to restore these constituents when given to *P. yoelii*-infected mice at the dose levels of 2.5 and 100 mg/kg, respectively, compared to mefloquine which does the same at 5.0 mg/kg dose. Methylene blue, like mefloquine also caused a rapid decline in percent parasitaemia, whereas menadione caused a delay in maturation of the infection, but could not cure the mice.

Abbreviations GSH: Glutathione · GCL: Glutamate cysteine ligase · GR: Glutathione reductase · Mf: Mefloquine · MB: Methylene blue · Md: Menadione

Introduction

Intracellular parasites, such as the malaria, require a highly efficient thiol metabolism to protect themselves from intracellular reactive oxygen species (ROS) and their derivatives (Docampo and Moreno 1984). This implies that they are highly susceptible to oxidative stress and this sensitivity is a promising target for drug action (Hunt and Stocker 1990; Schirmer et al. 1987). The role of oxidative stress as an important mechanism for the destruction of parasites and tumour cells is particularly well illustrated by many congenital and acquired factors that generate oxidative stress in human erythrocytes and offer partial protection against malaria (Hunt and Stocker 1990). Glutathione (GSH) is the central constituent of the collective antioxidant defenses in a cell, and plays an important role in the detoxification of electrophiles and maintaining the redox balance of the parasite and host–parasite interaction. Strategies for pharmacologically maintaining or increasing tissue GSH levels have received considerable interest in recent years in many of the diseases including malaria. Epidemiological, clinical observations and experimental evidences suggest that malarial parasites are more sensitive to oxidative stress than their host cells (Hunt and Stocker 1990; Golenser et al. 1991). The potential importance of the host cell GSH system for the survival of blood stage Plasmodia forms, has been discussed for decades (Ginsburg and Atamna 1994).

Since GSH of malarial parasites has been considered to be a drug target, it is therefore equally important to assess the modulation in the levels of the enzymes involved in GSH homeostasis of the affected host's tissues, that is, glutamate cysteine ligase (GCL) and glutathione reductase (GR). Methylene blue (MB), the first chemotherapeutic agent to be successfully used in humans (Vennerstrom et al. 1995), is known to interfere with the GSH metabolism of malaria-parasitised erythrocytes (Kelner and Alexander 1985). Menadione (Md), on the other hand, is a quinone-containing compound that has been utilized generally as a model for studies of oxidative damage. The present study describes the alteration in GSH content and the activities of the two key enzymes, that is, GCL and GR in hepatic and splenic tissues of *Plasmodium yoelii nigeriensis*-infected mice and their restoration after the treatment with MB and Md, and the results are compared with the standard antimalarial agent Mf.

K. Arora · A. K. Srivastava (✉)
Division of Biochemistry, Central Drug Research Institute, Lucknow, 226 001, India
E-mail: drarv1955@yahoo.com
Tel.: +91-522-2212411
Fax: +91-522-2223938

Materials and methods

Chemicals

Tris, phosphoenol pyruvate, sodium L-glutamate, L-α-aminobutyric acid, sodium EDTA, β-nicotinamide adenine dinucleotide (reduced), adenosine-triphosphate, mefloquine, menadione sodium bisulphite, methylene blue, saponin, BSA and PMSF were purchased from Sigma Chemical Co., USA. Oxidised and reduced GSH, β-nicotinamide adenine dinucleotide phosphate (reduced), Folin Ciocaltaeu's phenol reagent, pyruvate kinase and lactate dehydrogenase were purchased from Sisco Research Laboratories, Mumbai, India. Giemsa stain was from Qualigens India Ltd. All other chemicals used were of analytical grade.

Plasmodium yoelii nigeriensis infection in Swiss albino mice

A derived multidrug resistant strain of rodent malaria parasites *P. yoelii nigeriensis* (MDR) has been employed for enzymic as well as chemotherapeutic studies. In vivo maintenance of *P. yoelii nigeriensis* were carried out using Swiss albino mice (*Mus musculus*, out-bred strain). The animals were housed in plastic cages with proper care according to the guidelines of our institutional animal house ethics committee; under standard laboratory conditions of temperature (22 ± 1°C), humidity (50–60%) and maintained on commercially available pellet diet supplemented with soaked grains. Water was provided ad libitum. Breeding colonies of animals were maintained under specific pathogen free (SPF) environment in standard housing condition. The laboratory isolates of *P. yoelii* were routinely maintained by serial blood passage in mice. For this, the infected blood from donor mice was collected through cardiac puncture and appropriately diluted with anticoagulant (sterile acid citrate dextrose, ACD: dextrose anhydrous (24.5 g), citric acid monohydrate (7.5 g), sodium citrate dihydrate (22.0 g) in 1 l triple distilled water) so as to obtain nearly 2×10^7 parasitised RBCs/ml of diluted sample. The recipient mice were inoculated with parasite by intraperitoneal passage of 0.5 ml volume. The course of parasitaemia was monitored daily by microscopic examination of Giemsa-stained thin blood smears.

Measurement of GSH content, GCL and GR levels in liver and spleen of mice infected with *P. yoelii nigeriensis*

Mice were divided into five groups, namely, normal (uninfected) control, infected (pymdr) control, mefloquine (Mf) treated, menadione (Md) treated and methylene blue (MB) treated. The drugs were pulverized to fine powder and a fine suspension of each was made in distilled water. The treatment groups were administered Mf, Md and MB (i.p.) at doses of 5.0, 100 and 2.5 mg/kg, respectively, twice daily from day 1 post-infection. Parasitaemia was monitored day 3 onwards in all the infected groups.

Three mice from each of the earlier-mentioned groups were sacrificed by cervical dislocation on day 4, and liver and spleen excised, perfused and homogenized in ice-cold buffered KCl (150 mM KCl, 50 mM Na_2HPO_4/KH_2PO_4, pH 7.4) using a Potter Elvehjem homogenizer fitted with a teflon pestle. For GSH measurement, the homogenate was prepared in sulfosalicylic acid. The homogenate was then subjected to subcellular fractionation. For the goal, the homogenate was centrifuged at 1,000 *g* for 15 min, 10,000 *g* for 30 min, and subsequently at 100,000 *g* for 60 min to obtain mitochondrial, post-mitochondrial and cytosolic fractions, respectively. The post-mitochondrial and cytosolic fractions were saved at 4°C and used as the source of GSH and the enzymes GCL and GR, respectively.

GSH was measured by a spectrophotometric method based on the reductive cleavage of DTNB. Different aliquots were mixed with 800 µl of 0.1 M potassium phosphate buffer (pH 8.0) containing 1 mM EDTA, and 50 µl of DTNB (1 mg/ml). The absorbance of the resultant yellow colour was measured at 410 nm.

GCL activity was determined following a coupled enzyme procedure, in which the rate of ADP formation in the presence of pyruvate kinase, LDH, PEP and NADH is obtained from the decrease in absorbance of NADH at 340 nm (Seelig and Meister 1985). Enzyme activity was monitored at 37°C using a Shimadzu double beam UV spectrophotometer in reaction mixtures (final volume 1.0 ml) containing 100 mM Tris–HCl buffer; pH 8.4, 150 mM KCl, 0.5 mM acivicin, 5 mM ATP, 2 mM PEP, 10 mM L-glutamate, 10 mM L-α-aminobutyrate, 20 mM $MgCl_2$, 2 mM Na_2EDTA, 0.2 mM NADH, 1.6 µg/µl pyruvate kinase and 1.6 µg/µl lactate dehydrogenase. L-α-Aminobutyrate was used as the amino acid acceptor rather than L-cysteine because of the marked tendency of the latter amino acid to undergo spontaneous oxidation to the disulphide. Reaction was initiated by the addition of enzyme protein. The absorbance at 340 nm was monitored for 5 min at 30 s intervals.

GR activity was determined by the method described by Carlberg and Mannervik (1985). All enzyme kinetics experiments were carried out at room temperature (37°C) using a Shimadzu double beam UV spectrophotometer. Enzyme activity was spectrophotometrically monitored from the rate of conversion of NADPH to NADP ($e_{340} = 6.2\ mM^{-1}\ cm^{-1}$). For each assay, the absorbance of NADPH in the reaction mixture was measured over a period of 5 min. Assays were carried out in 1 ml of the following solution: 100 mM potassium phosphate buffer, pH 7.0; 1 mM EDTA; 1 mM GSSG; 0.2 mM NADPH and enzyme protein. Enzyme aliquots were preincubated in the presence of NADPH for 5 min in order to trace the non-enzymatic oxidation, after which GSSG was added to start the reaction. To correct

for non-specific oxidation of NADPH, the blank cuvette contained all the assay components except GSSG.

A unit of enzyme activity was expressed as the amount that catalyses the consumption of 1 µmol of substrate per minute, using a molar extinction coefficient of 6.2 mM^{-1} cm^{-1} for NADH/NADPH. Protein was estimated in the crude homogenate/sub-cellular fractions following the method of Lowry et al. (1951) using BSA as standard. The percent protection offered by the drugs was calculated.

Statistical analysis

In animal experimentation, the results were expressed in terms of mean ± standard deviation (SD). Groups were compared by one-way analysis of variance (ANOVA) using prism software. Significance differences were set at $P < 0.05$, $P < 0.005$ and $P < 0.001$, respectively.

Results

Swiss albino mice were infected with *P. yoelii nigeriensis* and the percentage infection or parasitaemia was ascertained as described in Materials and methods. Figure 1a is a depiction of the rise in parasitaemia (*P. yoelii*) and percent mortality of mice with time in the infected control mice. A progressive increase in percent parasitaemia was obtained, with a maximum of 44% parasitaemia by day 8. The percent mortality was found to be maximum by day 10 (75%).

Figure 1b shows the effect of Mf, Md and MB treatment on percent parasitaemia in *P. yoelii*-infected mice. Results obtained show that Mf cured the parasitaemia of mice by the 6th day. On the other hand, MB did not allow parasitaemia to mature beyond 6%, and cured the mice by the 6th day, while Md caused a delay in maturation of infection, but could not cure the mice.

Hepatic and splenic GSH levels and GCL and GR activity profile during *P. yoelii* infection in Swiss mice has been shown in Figs. 2, 3 and 4. The results obtained show that the activity of GCL and GR in all cases decreased with rise in parasitaemia. Mf, Md and MB treatment caused restoration of the levels of the two enzymes. Mf caused 100% protection on both hepatic and splenic GCL and GR activities. Md, on the other hand, protected hepatic GCL by 97.9% and all others by about 100%. MB caused protection to the tune of 25 and 31% on hepatic and splenic GCL, respectively, and 100 and 69.6% on hepatic and splenic GCL, respectively.

Mf offered 50–100% and 35–100% protection on hepatic and splenic GSH levels of *P. yoelii* infected mice, while Md caused 73.44% protection on hepatic GSH levels and 100% protection on splenic GSH levels. MB protected hepatic and splenic GSH levels by 80.4 and 90.7%, respectively.

Discussion

The present study indicates that *P. yoelii nigeriensis* infection causes a decrease in the hepatic and splenic GSH levels and both GCL and GR activities as compared to the normal tissues. Mefloquine (Mf), a quinoline methanol derivative is related structurally to quinine. The compound was found effective against malaria, resistant to other forms of treatment when first introduced and because of its long half-life was a good prophylactic, but widespread resistance has now developed against Mf alone. It is now being used in combination with other drugs like artemesins. Mf is known to block the degradation of heme in aqueous solution by GSH (Famin et al. 1999). In the present study, Mf cured the mice of infection and its treatment also caused a restoration in the levels of GSH as well as that of both GCL and GR.

Menadione (2-methyl-1,4-naphthoquinone, vitamin K, Md) is a quinone-containing compound that has been utilized generally as a model for studies of oxidative damage. Md may cause a decrease in intracellular GSH level by three different mechanisms (DiMonte et al. 1984). First, by oxidation of GSH to GSSG; during the

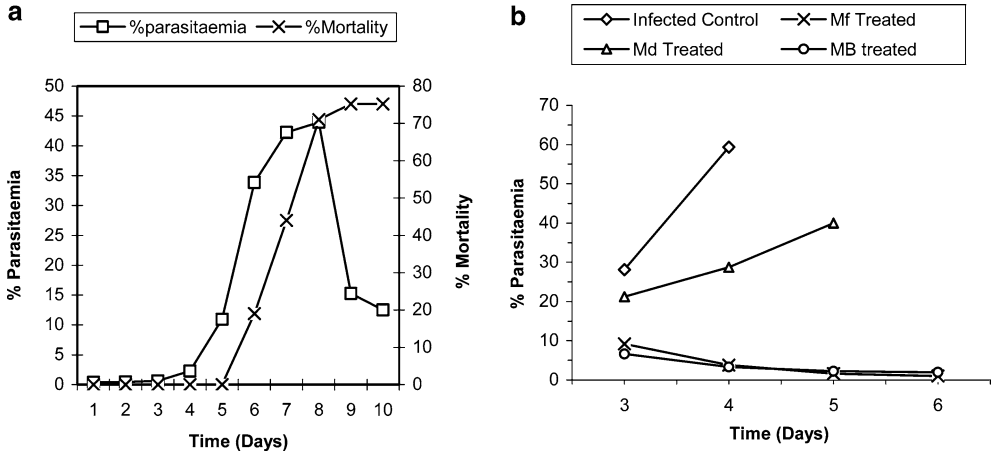

Fig. 1 Graphs showing **a** rise in parasitaemia and mortality with time in normal uninfected mice, and **b** effect of mefloquine, menadione and methylene blue treatment on parasitaemia in *P. yoelii* infected mice. Dose: mefloquine 5 mg/kg, menadione 100 mg/kg, methylene blue 2.5 mg/kg intraperitoneally, twice, daily

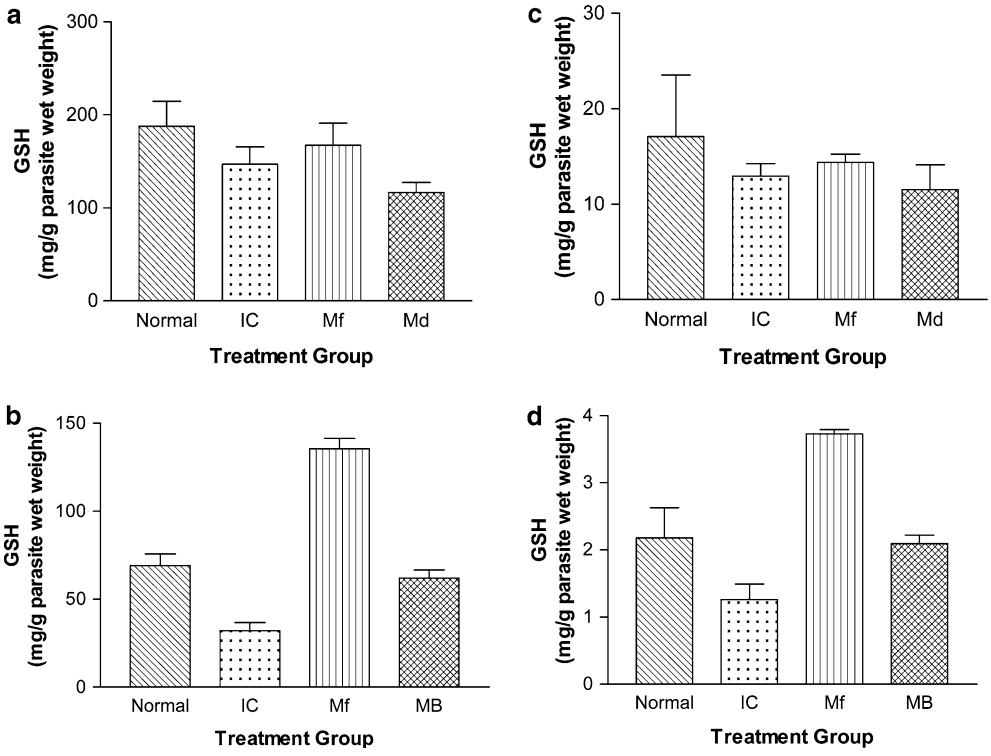

Fig. 2 Hepatic (**a**, **b**) and splenic (**c**, **d**) GSH levels of *P. yoelii* mdr infected Swiss albino mice after mefloquine and menadione/methylene blue treatment. Values are mean ± SD of three separate observations; $P < 0.05$ with respect to normal. IC, infected control; Mf, mefloquine treated; Md, menadione treated; MB, methylene blue treated

one-electron reduction of Md to the semiquinone radical, and its subsequent reoxidation by oxygen as well as by the direct interaction of Md with intracellular thiols, $\{O_2\bullet^-$ is produced. Dismutation of $\{O_2\bullet^-$ yields H_2O_2, which can be further metabolised to H_2O by the GPx system at the expense of GSH (Wendel 1981). Most of the depletion is through this mechanism. Second, by the formation of GSH–Md conjugate. Md is conjugated with GSH to yield 2-methyl-3- *S*-glutathionyl-1,4-naphthoquinone (thiodione) (Akerboom et al. 1988;

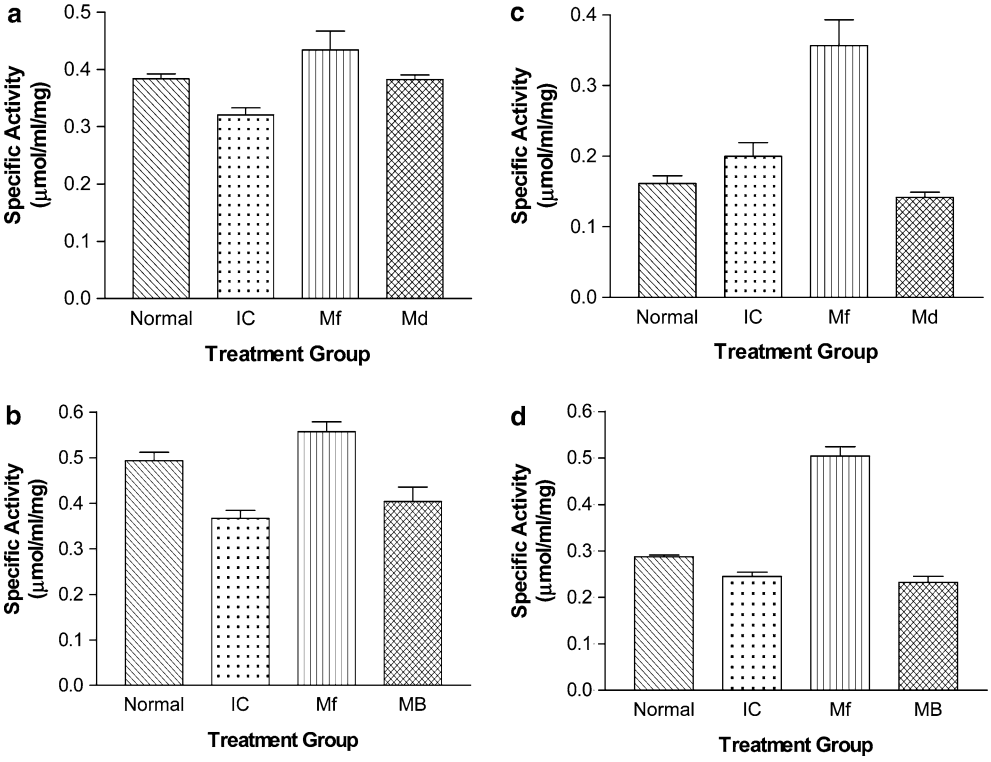

Fig. 3 Hepatic (**a**, **b**) and splenic (**c**, **d**) GCL activity profile of *P. yoelii* mdr infected Swiss albino mice after mefloquine and menadione/methylene blue treatment. Values are mean ± SD of three separate observations; $P < 0.05$ with respect to normal. IC, infected control; Mf, mefloquine treated; Md, menadione treated; MB, methylene blue treated

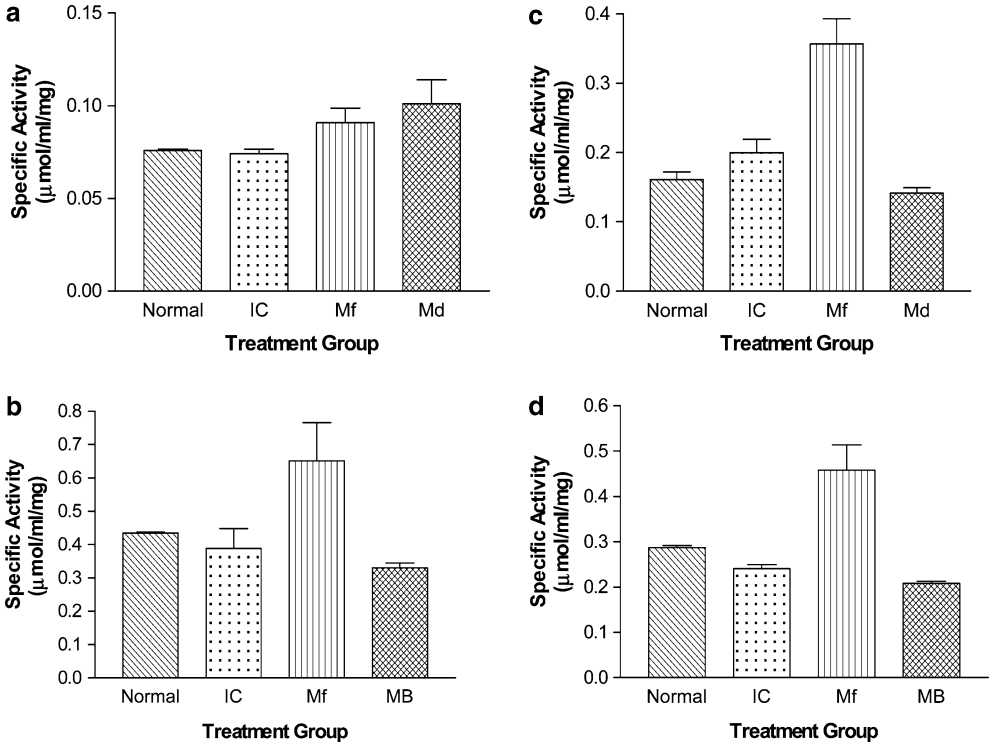

Fig. 4 Hepatic (**a**, **b**) and splenic (**c**, **d**) GR activity profile of *P. yoelii* mdr infected Swiss albino mice after mefloquine and menadione/methylene blue treatment. Values are mean ± SD of three separate observations; $P < 0.05$ with respect to normal. IC, infected control; Mf, mefloquine treated; Md, menadione treated; MB, methylene blue treated

Nickerson et al. 1963). Third, GSH–protein mixed disulphides are also formed as a result of Md metabolism.

Menadione has been used for various clinical purposes to prevent vitamin K deficiencies, myopathies and to treat malaria (Meister 1991; Gasser 1959; Eleff et al. 1984; Lopez-Shirley et al. 1994). Treatment with this compound caused a restoration in the levels of GSH as well as that of the two enzymes in the present investigation. It caused a delay in infection maturation, but could not cure the mice completely.

Methylene blue (MB), an antimalarial compound, the first chemotherapeutic agent to be successfully used in humans (Vennerstrom et al. 1995) is known to interfere with the GSH metabolism of parasitised erythrocytes (Kelner and Alexander 1985). *P. falciparum* GR, which has emerged as a drug target against tropical malaria, can be inhibited by the antimalarial drug MB (Färber et al. 1998). MB stimulates the oxidation of GSH in RBCs in vitro and in vivo. This oxidation has been attributed to hydrogen peroxide that is generated from the auto oxidation of leukomethylene blue arising from the reduction of MB by NADPH. The reducing power of MB appears to be derived from its reaction with NADPH because MB therapy is ineffective in patients with glucose-6-phosphate dehydrogenase deficiency, the enzyme which is the primary source of NADPH in the red blood cell. In addition, decreases in GSH have been noted after MB administration (Goldstein 1974; Harvey and Keitt 1983) in vivo and after the in vitro incubation of RBCs with MB (Jacob and Jandl 1966). This GSH decrease has been attributed to competition of MB with GSSG for NADPH thereby inhibiting the conversion of GSSG to GSH and the oxidation of GSH by hydrogen peroxide that is generated from NADPH, MB, and oxygen (Harvey and Keitt 1983; Jacob and Jandl 1966). Standard redox potentials (White et al. 1978) indicate that MB may directly oxidize GSH. In general, the mechanism of action of MB appears to be an activation of otherwise dormant NADPH linked reductase system in red cell. MB treatment restored the GSH levels as well as GCL and GR activities and also cured the mice of parasitaemia.

Oxidant stress is often invoked as a mechanism for the inhibition of malarial parasite growth in vivo. The

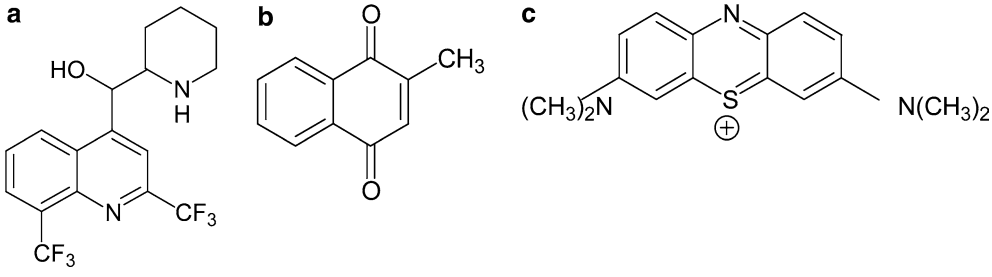

Fig. 5 Chemical formulae of **a** mefloquine, **b** menadione, **c** methylene blue

depletion of reduced GSH, a protective substance that is also a cofactor in some enzymatic processes, may result in cell injury and parasite death (Fairfield et al. 1983; Roth et al. 1984). MB and Md treatment imposed oxidant stress upon the malaria parasite, thus causing a decline in the percent parasitaemia in these groups.

Therefore, it can be concluded that malaria infection can depress the hepatic and splenic GSH levels and GCL and GR functions, and that these are normalized within a week after treatment with the antimalarials mefloquine, methylene blue and menadione. Also, mefloquine and methylene blue were successful in curing the mice, causing a rapid decline in percent parasitaemia, while menadione caused a delay in maturation of infection, but could not cure the mice.

Acknowledgements Financial assistance in the form of Senior Research Fellowship to one of our authors (KA) from CSIR, New Delhi (India) and Volkswagen Stiftung, Hannover (Germany) in the form of ad hoc research grant to AKS for carrying out this research are gratefully acknowledged.

References

Akerboom T, Bultmann T, Sies H (1988) Inhibition of taurocholate excretion during menadione metabolism in the perfused rat liver. Arch Biochem Biophys 263:10–18

Carlberg I, Mannervik B (1985) Glutathione reductase. Methods Enzymol 113:484–490

DiMonte D, Ross D, Bellomo G, Eklöw L, Orrenuis S (1984) Alterations in intracellular thiol homeostasis during the metabolism of menadione by isolated rat hepatocytes. Arch Biochem Biophys 235:334–342

Docampo R, Moreno SNJ (1984) Free radical metabolites in the mode of action of chemotherapeutic agents and phagocytic cells on *Trypanosoma cruzi*. Rev Infect Dis 6:223–228

Eleff S, Kenneway NG, Buist NRM, Darley-Usmar VM, Capaldi RA, Bank WJ, Chance B (1984) ^{31}P-NMR study of improvement in oxidative phosphorylation by vitamin K_3 and C in a patient with a defect in electron transport at complex III in skeletal muscles. Proc Natl Acad Sci 81:3529–3533

Fairfield AS, Meshnick SR, Eaton JW (1983) Malarial parasites adopt host red cell superoxide dismutase. Science 221:764

Famin O, Krugliak M, Ginsburg H (1999) Kinetics of inhibition of glutathione-mediated degradation of ferriprotoporphyrin IX by antimalarial drugs. Biochem Pharmacol 58:59 –68

Färber PM, Arscott LD, Williams CH Jr, Becker K, Schirmer H (1998) Recombinant *Plasmodium falciparum* glutathione reductase is inhibited by the antimalarial dye methylene blue. FEBS Lett 422:311–314

Gasser C (1959) Heinz body anemia and related phenomena. J Paediatr 54:673–690

Ginsburg H, Atamna H (1994) The redox status of malaria-infected erythrocytes: an overview with an emphasis on unresolved problems. Parasite 1:5–13

Goldstein BD (1974) Exacerbation of dapsone-induced Heinz body hemolytic anemia following treatment with methylene blue. Am J Med Sci 267:291–297

Golenser J, Marva E, Chevion M (1991) The survival of *Plasmodium* under oxidant stress. Parasitol Today 7:142–146

Harvey JW, Keitt AS (1983) Studies of the efficacy and potential hazards of methylene blue therapy in aniline-induced methaemoglobinaemia. Br J Haematol 54:29–41

Hunt NH, Stocker R (1990) Oxidative stress and the redox status of malaria-infected erythrocytes. Blood Cells 16:499–530

Jacob HS, Jandl JH (1966) Effects of sulfhydryl inhibition on red blood cells. III Glutathione in the regulation of hexose monophosphate pathway. J Biol Chem 241:4243–4250

Kelner MJ, Alexander NM (1985) Methylene blue directly oxidizes glutathione without the intermediate formation of hydrogen peroxide. J Biol Chem 260:15168–15171

Lopez-Shirley K, Zhang F, Gosser D, Scott M, Meshnick SR (1994) Antimalarial quinones: redox potential dependence of methemoglobin formation and hence release in erythrocytes. J Lab Clin Med 123:126–130

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–276

Meister A (1991) Glutathione deficiency produced by the inhibition of its synthesis, and its reversal: Applications in research and therapy. Pharmacol Therap 51:155–194

Nickerson WJ, Falcone G, Strauss G (1963) Studies on the quinone-thioesters I. Mechanism of formation and properties of thiodione. Biochemistry 2:537–543

Roth EF Jr, Raventos-Suarez C, Gilbert H, Stump D, Tanowitz H, Rowin KS, Nagel RL (1984) Oxidative stress and falciparum malaria: a critical review of the evidence. In: Eaton J, Brewer G (eds) Malaria and the red cell. Alan R. Liss, New York, p 35

Schirmer RH, Schollhammer T, Eisenbrand G, Krauth-Siegel RL (1987) Oxidative stress as a defence mechanism against parasitic infections. Free Radic Res Commun 3:3–12

Seelig GF, Meister A (1985) Glutathione biosynthesis: γ-glutamylcysteine synthetase from rat kidney. Methods Enzymol 113:379–392

Vennerstrom JL, Makler MT, Angerhofer CK, Williams JA (1995) Antimalarial dyes revisited: Xanthenes, azines, oxazines and thiazines. Antimicrob Agents Chemother 39:2671–2677

Wendel A (1981) In: Jakoby WB (ed) Enzymatic basis of detoxification. Academic Press, New York, pp 333–353

White A, Handler P, Smith EL, Hill RL, Lehman IR (1978) Principles of biochemistry. 6th edn., pp. 112–114, 145–146