Plasmodium falciparum-infected red blood cells depend on a functional glutathione *de novo* synthesis attributable to an enhanced loss of glutathione

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During the erythrocytic cycle, *Plasmodium falciparum* is highly dependent on an adequate thiol status for its survival. Glutathione reductase as well as *de novo* synthesis of GSH are responsible for the maintenance of the intracellular GSH level. The first and rate-limiting step of the synthetic pathway is catalysed by γ-glutamylcysteine synthetase (γ-GCS). Using L-buthionine-(*S*,*R*)-sulphoximine (BSO), a specific inhibitor of the γ-GCS, we show that the infection with *P. falciparum* causes drastic changes in the GSH metabolism of red blood cells (RBCs). Infected RBCs lose GSH at a rate 40-fold higher than non-infected RBCs. The *de novo* synthesis of the tripeptide was found to be essential for parasite survival. GSH depletion by BSO inhibits the development of *P. falciparum* with an IC_{50} of 73 μM. The effect of the drug is abolished by supplementation with GSH or GSH monoethyl ester. Our studies demonstrate that the plasmodicidal effect of the inhibitor BSO does not depend on its specificity towards its target enzyme in the parasite, but on the changed physiological needs for the metabolite GSH in the *P. falciparum*-infected RBCs. Therefore the depletion of GSH is proposed as a chemotherapeutic strategy for malaria, and γ-GCS is proposed as a potential drug target.

Key words: buthionine-(*S*,*R*)-sulphoximine, chemotherapy, γ-glutamylcysteine synthetase, malaria.

INTRODUCTION

Plasmodium falciparum is the causative agent of malaria tropica, the most prevailing parasitic disease worldwide, with 300–500 million clinical cases and 1.5–2.7 million deaths per year. The spread of drug resistance is a continuing health problem and raises the need for the development of new antimalarial drugs [1].

Clinical observations and experimental evidence indicate that, during schizogony, *P. falciparum* and its host red blood cell (RBC) are exposed to enhanced oxidative stress, mainly due to parasitic haemoglobin digestion and the host immune response [2,3]. Although the RBC, as well as the parasite, is supplied with antioxidative systems [4,5], it was shown that the balance of pro- and anti-oxidants in the parasite–host unit is very fragile and susceptible to additional exogenous oxidative stress [6–8].

The tripeptide GSH is the predominant low-molecular-mass thiol in most cells and functions in the maintenance of the intracellular redox state, the generation of DNA precursors and the detoxification of xenobiotics and reactive oxygen species [9]. It is also part of the GSH redox cycle, an important endogenous protective system against oxidative stress, in which peroxides are detoxified by glutathione peroxidase [10]. During this reaction GSH is oxidized to GSSG, which is re-generated by the NADPH-dependent glutathione reductase (GR) [11]. Inhibition of the GSH redox cycle, either by drugs against GR [12,13] or attributable to inherited glucose 6-phosphate dehydrogenase deficiency [14,15], impairs the development of the erythrocytic stages of *P. falciparum*. These studies suggest that the antioxidant GSH plays a critical role for parasite survival.

Whereas the GR reaction is the main pathway for maintaining intracellular GSH levels in RBCs under normal conditions, in cases of oxidative challenge the *de novo* synthesis of GSH becomes more important [16,17]. GSH is synthesized by a distinct two-step biosynthetic pathway. The first and rate-limiting step is represented by the γ-glutamylcysteine synthetase (γ-GCS), which catalyses the ligation of L-glutamate and L-cysteine [9]:

$$\text{L-Glutamate} + \text{L-cysteine} + \text{ATP} \xrightarrow{\gamma\text{-GCS}} \text{L-}\gamma\text{-glutamyl-L-cysteine} + \text{ADP} + \text{P}_\text{i}$$

We have recently cloned the gene for the γ-GCS from *P. falciparum* [18]. The predicted molecular mass of the deduced protein is unusual, owing to three large insertions. In addition, within the first insertion, *P. falciparum* γ-GCS contains a conspicuous octameric tandem repeat. Since this repeat varies in number between different *P. falciparum* strains, the corresponding molecular masses of the putative γ-GCS range from 124 to 133 kDa. In contrast, the catalytic subunit of the mammalian enzyme is only 70 kDa [19]. In trypanosomatids γ-GCS was also shown to contain an insertion which increases the molecular mass of the protein to 77.4 kDa. It has been hypothesized that the insertion in *Trypanosoma brucei* may fulfil the functions of the regulatory subunit of the mammalian protein [20].

The specific transition-state inhibitor of γ-GCS L-buthionine-(*S*,*R*)-sulphoximine (BSO) [21] was successfully used to deplete GSH in various mammalian tissues and cell lines [22,23] as well as in the parasite *Trypanosoma brucei* [24]. In tumour cells, BSO treatment leads to an increased sensitivity to oxidative stress, radiation and drugs [22,23]. Recently, phase I studies showed the low toxicity of BSO administration to normal tissues [23]. In this study we used BSO and exogenously administrated GSH and GSH monoethyl ester to investigate GSH metabolism in *P. falciparum*-infected RBCs. Our data show that inhibition of γ-GCS by BSO has an antimalarial effect due to an enhanced loss of GSH in infected RBCs. We propose the γ-GCS from *P. falciparum* as a potential target for malaria chemotherapy.

Abbreviations used: BSO, L-buthionine-(*S*,*R*)-sulphoximine; EBBS, Earle's balanced salt solution; γ-GCS, γ-glutamylcysteine synthetase; GR, glutathione reductase; mBBr, monobromobimane; OPA, *o*-phthalaldehyde; RBC, red blood cell; SLO, streptolysin-O; TRBC, trophozoite-infected RBC.

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EXPERIMENTAL

Chemicals

L-[U-^{14}C]Glutamic acid (264 mCi·mmol^{-1}) was purchased from Amersham–Buchler (Braunschweig, Germany). Acetonitrile, L-alanine and saponin were obtained from Serva (Heidelberg, Germany). Cysteinylglycine, GSH monoethyl ester and γ-glutamylaminobutyrate were from Bachem (Heidelberg, Germany). Monobromobimane (mBBr) and *o*-phthalaldehyde (OPA) were obtained from Fluka (Neu-Ulm, Germany). All other chemicals used were from Sigma (Deisenhofen, Germany). Streptolysin-O (SLO) was kindly provided by Professor S. Bhakdi, Institute of Medical Microbiology and Hygiene, Johannes-Gutenberg-University, Mainz, Germany.

Parasites

P. falciparum FCBR-strain (Colombia) was maintained as a synchronous continuous culture in human RBCs (blood group A$^+$), RPMI 1640 medium supplemented with 25 mM Hepes, 20 mM sodium bicarbonate, 40 μg/ml gentamicin and 10 % (v/v) heat-inactivated human blood-group-A$^+$ plasma at 5 % (v/v) haematocrit [25]. Petri dishes and flasks were gassed with $N_2/O_2/CO_2$ (18:1:1) and incubated at 37 °C with daily changes of medium. The percentage of infected RBCs and the development of the culture were determined by optical-microscopical evaluation of Giemsa-stained thin smears. Synchronization was performed by incubation of cells in 2 vol. of 0.3 M alanine, 10 mM Hepes, pH 7.4, for 5 min at 37 °C [26]. To obtain pure trophozoite-infected RBCs (TRBCs), a culture of 5–10 % parasitaemia of the trophozoite stage was enriched by treatment with Gelafundin (a kind of gelatin supplied by Braun Melsungen, Melsungen, Germany) to about 60–80 % [27]. Subsequently, the TRBCs were separated from the remaining non-infected RBCs by a discontinuous Percoll/alanine gradient centrifugation [28]. The TRBC fraction was either harvested or after washing in RPMI 1640 medium without plasma was allowed to recover for 1 h under standard culture conditions before used in further experiments. The number of TRBCs was determined in a cell counter (Coulter Max M; Coulter Electronics, Krefeld, Germany). For compartmental analysis, parasites were isolated from the RBC either by saponin lysis [29] or by using the pore-forming toxin SLO [30]. For saponin lysis, TRBCs at a haematocrit of 25 % were incubated for 10 min at 4 °C in Earle's balanced salt solution (EBSS) containing 0.15 % (w/v) saponin before adding 4 vol. of EBSS. For SLO treatment 2×10^8 TRBCs were diluted in EBSS to a haematocrit (v/v) of 10 % and were incubated for 6 min at 20 °C with 1.43 μg of the pore-forming toxin. Subsequently, both reaction mixtures were centrifuged at 1500 ***g*** for 5 min at 4 °C. The supernatants were used for analysing the GSH contents in the erythrocytic cytoplasm, whereas the isolated parasites in the pellet were washed twice in ice-cold EBSS before further investigation.

Determination of the effects of BSO, GSH and GSH monoethyl ester on the survival of *P. falciparum* in culture

Drug testing was performed in duplicate in 24-well culture plates starting with the synchronized ring stage (6–12 h) culture at 0.5–1.0 % parasitaemia. Increasing concentrations of BSO (0–500 μM) were added to the *P. falciparum* culture and the effect on the growth and development of the parasites was investigated. In addition, the influence of the simultaneous administration of BSO and 1 mM GSH or 1 mM GSH monoethyl ester was determined. The medium and the drugs were changed daily. Parasitaemia was estimated microscopically by Giemsa-stained thin smears taken after 24, 48 and 72 h, the number of infected RBCs per 1000 RBCs being counted . The IC_{50} values were determined graphically in terms of concentration of BSO versus percentage of growth inhibition.

Effect of BSO, GSH and GSH monoethyl ester on GSH level and γ-GCS activity

RBCs and synchronized *P. falciparum* culture (about 10 % parasitaemia; ring stage) were treated with different non-lethal BSO concentrations for 24 h under standard culture conditions. TRBCs were separated from the non-infected RBCs on Gelafundin and Percoll/alanine gradients as described above to investigate the dose-dependent effect of the drug on GSH level and γ-GCS activity.

Aliquots of pure TRBCs were cultured for 5 h, and 100 μM BSO was added at different time points. Subsequently, GSH levels and γ-GCS activities were determined.

Further, for compartmental studies, Gelafundin-enriched TRBCs were incubated with 100 μM BSO for 3 h and then purified on a Percoll/alanine gradient. GSH levels and γ-GCS activities were measured in the TRBC fraction and also in the host-cell and parasite compartments after saponin lysis and SLO lysis respectively.

Gelafundin-enriched TRBCs were incubated with or without 100 μM BSO for 3 h under standard culture conditions. GSH (1 mM or 15 mM) or GSH monoethyl ester (1 mM or 15 mM) were added to the BSO-pretreated cultures, which were cultivated for an additional 2 h. The thiol levels in the TRBCs were monitored over 5 h to investigate the effect of exogenous thiols.

In order to investigate whether BSO had an effect on the transcription level of the γ-GCS gene, a Northern-blot analysis was performed with RNA isolated from TRBCs incubated with BSO for 24 h and untreated control TRBCs as described in [18].

Thiol pattern and GSH level of *P. falciparum*

Low-molecular-mass thiols in RBCs, TRBCs and isolated *P. falciparum* were analysed as mBBr derivatives by reversed-phase HPLC. The samples were prepared by the method of Fiskerstrand et al. [31], with minor modifications. A 10 μl portion of RBCs, TRBCs or isolated *P. falciparum* cells [$(2.5–5.0) \times 10^7$ cells] in 1.5 ml Eppendorf tubes placed on ice/water were supplemented with 5 μl of 1 M $NaBH_4$ in 0.066 M NaOH and 33 % (v/v) DMSO, 3 μl of 2 mM EDTA and 1.65 mM dithiothreitol, 3 μl of octan-1-ol and 7 μl of 1.8 M HCl. After 3 min (to allow the reduction of the disulphides), 35 μl of 1 M ethylmorpholine buffer, pH 8.5, 67 μl of water and 7 μl of 25 mM mBBr were added. The derivatization was carried out at 20 °C for 10 min in the dark and was terminated by adding 13 μl of 100 % acetic acid. After 20 min on ice in the dark the sample was extracted with 200 μl of dichloromethane and centrifuged for 2 min at 10000 ***g***. The water-soluble phase was injected on to an analytical HPLC column packed with Spherisorb ODS II (5 μm; end capping; Machery–Nagel, Düren, Germany) and separated by the method of Zängerle et al. [32] with minor modifications. The column was eluted at a flow rate of 0.55 ml/min by the following gradient of solvent A (0.25 % acetic acid) and solvent B (100 % acetonitrile): 0 min, 0 %; 10 min to 45 min, 8 %; 70 min, 15 %; 85 min, 55 %. The effluent was monitored by a fluorescence spectrophotometer (excitation 400 nm; emission 475 nm) (SFM 25; Kontron, Eching, Germany). Cysteine, cysteinylglycine, glycylcysteine, homocysteine and GSH were used as standards. Other standards used were ovothiol A, cystamine, cysteamine, glutathionylspermidine and N^1,N^8-bis(glutathionyl)spermidine.

For the determination of GSH levels in non-infected RBCs and TRBCs, an alternative method was used [33]. One volume (minimum 5×10^7 cells) of RBCs or TRBCs was added to two volumes of 5 % (w/v) sulphosalicylic acid and mixed for 1 min. The samples were centrifuged at 4 °C for 15 min at 10 000 ***g***. The supernatant was added to 700 μl of 143 mM sodium phosphate (pH 7.5)/6.3 mM EDTA/0.34 mM NADPH, 100 μl of 6 mM 5,5′-dithiobis-(2-nitrobenzoate) and water was added to 950 μl. The mixture was incubated for 20 min at 20 °C and the reaction was started by adding 50 μl GR (20 units·ml^{-1}). The concentration of GSH was determined by observing the rate of change in absorption at 412 nm in a spectrophotometer (Uvikon 930; Kontron). Concentrations of GSH (or GSSG) were calculated relative to known standard concentrations of either peptide.

γ-GCS assay

The assay for the γ-GCS was performed as previously described [34]. The 100 μl reaction mixture contained 0.1 M Tris/HCl, pH 8.0, 20 mM $MgCl_2$, 2 mM EDTA, 1 mM [^{14}C]glutamate (25 nCi), 10 mM L-α-aminobutyrate, 10 mM ATP, 5 mM creatine phosphate, 1 unit of creatine phosphokinase and the cell lysate of $(1.0–1.5) \times 10^8$ RBCs, TRBCs or isolated *P. falciparum* cells. The cell lysate was supplemented with a protease inhibitor cocktail [4-(2-aminoethyl)benzenesulphonyl fluoride, pepstatin A, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane ('E-64'), bestatin, leupeptin and aprotinin] according to the manufacturer's recommendations (Sigma). Following incubation at 37 °C for 20 min, the reaction was terminated by the addition of 10 μl of 2 M $HClO_4$ and heating for 3 min at 95 °C. After cooling on ice, the samples were centrifuged at 10000 ***g*** for 5 min. The product of the enzyme reaction, γ-glutamylaminobutyrate, was determined by reversed-phase HPLC or TLC. For the analysis by reversed-phase HPLC, 50 μl of the samples saturated with sodium bicarbonate were derivatized with OPA-reagent (1:4, v/v) and subjected to a Spherisorb ODS II 5 μm-particle-size column (0.4 cm × 12 cm; Knauer, Berlin, Germany). The OPA reagent is composed of 40 mM OPA, 0.1 M sodium borate, 0.14 M 2-mercaptoethanol. The mobile phase consists of solvent A [50 mM NaH_2PO_4 (pH 5.5)/methanol (4:1, v/v)] and solvent B [50 mM NaH_2PO_4 (pH 5.5)/methanol (1:4, v/v)]. Separation was performed at a flow rate of 1 ml·min^{-1} with the following gradient (% of solvent B): 0 min, 0 %; 10 min, 10 %; 25 min, 55 %).

The separation of the γ-GCS product by TLC was performed on cellulose F plastic sheets (20 cm × 20 cm; 0.1 mm thickness; Merck, Darmstadt, Germany) using a mobile phase of butan-1-ol/acetic acid/water (12:3:5, by vol.).

γ-Glutamylaminobutyrate was revealed on the TLC sheets by autoradiography, the spots were cut out and the radioactivity was measured in 3 ml of Packard Pico Fluor 30 liquid-scintillation cocktail. Counting efficiency was 80–82 % in the Packard TriCarb 2000 liquid-scintillation counter (Canberra-Packard, Dreieich, Germany). The enzyme activity was determined relative to cell number.

Efflux of glutathione from non-infected RBCs and TRBCs

Non-infected RBCs or TRBCs were washed in prewarmed EBSS. Subsequently the cells were diluted to 10 % haematocrit in EBSS containing 5 mM glucose and incubated for up to 150 min. At different time points 500 μl aliquots were withdrawn, centrifuged at 3000 ***g*** at 4 °C for 3 min and 400 μl of the supernatant was used to determine the GSH content as described by Tietze [33]. GSH standards (0.5–4 μM) were treated the same way as the samples.

RESULTS

Thiol pattern of *P. falciparum*

In isolated red-blood-cell stages of *P. falciparum*, GSH was found to be the major low-molecular-mass thiol, with 7.04 ± 0.31 nmol/mg of protein, compared with the cysteine concentration of 0.45 ± 0.03 nmol/mg of protein (Figure 1). The other low-molecular-mass thiol (peak A, at approx. 20 min) detected in the parasites could not be assigned to any of the standard thiols used, and further analysis is required to elucidate the identity of this peak.

γ-GCS activity and GSH distribution in the parasite–host unit

Compartmental analysis performed after either saponin lysis or SLO treatment revealed that the specific γ-GCS activity is higher in the host cell cytosol (106.6 ± 5.3 nmol·min^{-1}·10^{10} cells^{-1}) than in the parasite (13.1 ± 2.6 nmol·min^{-1}·10^{10} cells^{-1}). Compared with non-infected RBCs the specific γ-GCS activity is

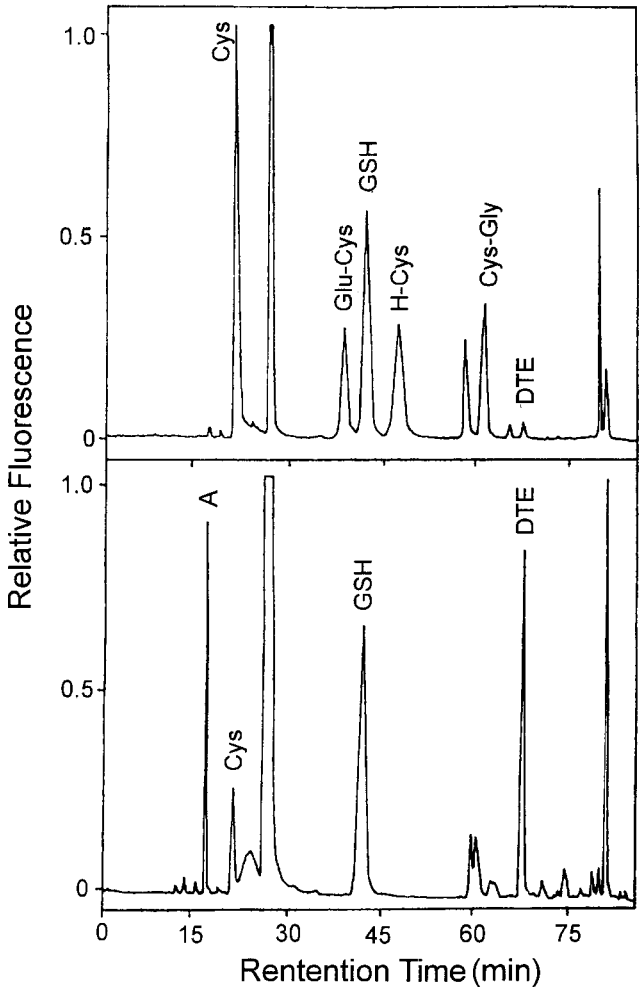

Figure 1 HPLC analysis of low-molecular-mass thiols of *P. falciparum*

(**A**) A mixture of mBBr-derivatized thiol standards separated by reversed-phase HPLC. A 100 pmol sample of cysteine (Cys) and 50 pmol of cysteinylglycine (Cys-Gly), glycylcysteine (Gly-Cys), GSH and homocysteine (H-Cys), respectively, were injected after reduction with DTE. (**B**) The thiol pattern of *P. falciparum* isolated from a mixed culture by saponin. The parasite contains an unidentified mBBr derivative (peak A). Derivatization and chromatographic conditions are described in the Experimental section.

Table 1 γ-GCS activity and GSH distribution in the host–parasite unit

Cell type	γ-GCS activity (nmol · min^{-1} · 10^{10} cells^{-1})* Control	3 h BSO†
TRBC	141.6 ± 4.7	34.8 ± 2.4
RBC cytosol	106.6 ± 5.3	23.3 ± 1.9
Parasite	13.1 ± 2.6	3.4 ± 0.4
RBC	187.7 ± 20.8	n.d.‡

Cell type	GSH content (μmol · 10^{10} cells^{-1})* Control	3 h BSO†
TRBC	0.89 ± 0.16	0.32 ± 0.04
RBC cytosol	0.66 ± 0.10	0.27 ± 0.03
Parasite	0.14 ± 0.05	0.05 ± 0.01
RBC	2.04 ± 0.06	n.d.‡

* Mean values ± S.D. for four separate experiments.
† Cells were treated with 100 μM BSO for 3 h.
‡ Not determined.

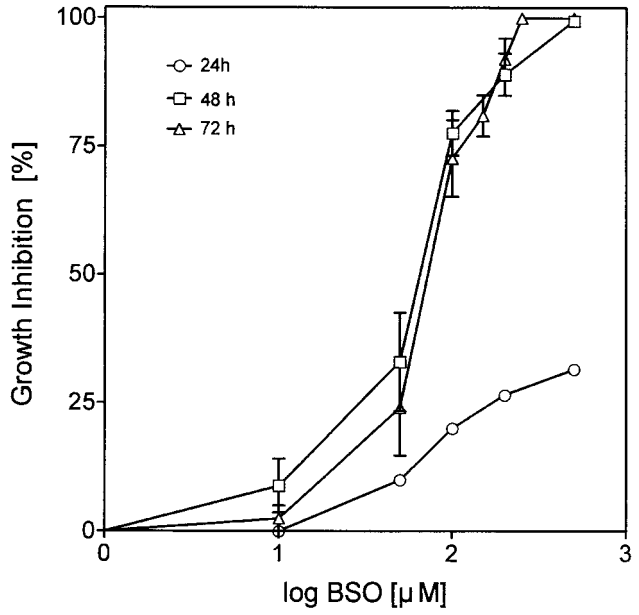

Figure 2 Effect of BSO on the proliferation of *P. falciparum* in *in vitro* culture

Drug screening was performed for 24 , 48 and 72 h as outlined in the Experimental section. Mean values ± S.D. for three separate experiments are given. Treatment, with increasing BSO concentrations, of a synchronous *P. falciparum* ring-stage (6–12 h) culture for 24 , 48 and 72 h. The IC_{50} for BSO was determined to be 73 μM after 48 h of treatment.

slightly decreased in TRBCs (187.7 ± 20.8 nmol · min^{-1} · 10^{10} cells^{-1} versus 141.6 ± 4.7 nmol · min^{-1} · 10^{10} cells^{-1}; Table 1).

The level of total GSH in TRBCs was determined to be 0.89 ± 0.16 μmol · 10^{10} cells^{-1}, which is about 44 % of the GSH content found in non-infected RBC (Table 1). Compartmental analysis revealed that most of the GSH is associated with the RBC cytoplasm, which contains 0.66 ± 0.10 μmol of GSH · 10^{10} cells^{-1}. The GSH level in the parasite compartment is 0.14 ± 0.05 μmol · 10^{10} cells^{-1}.

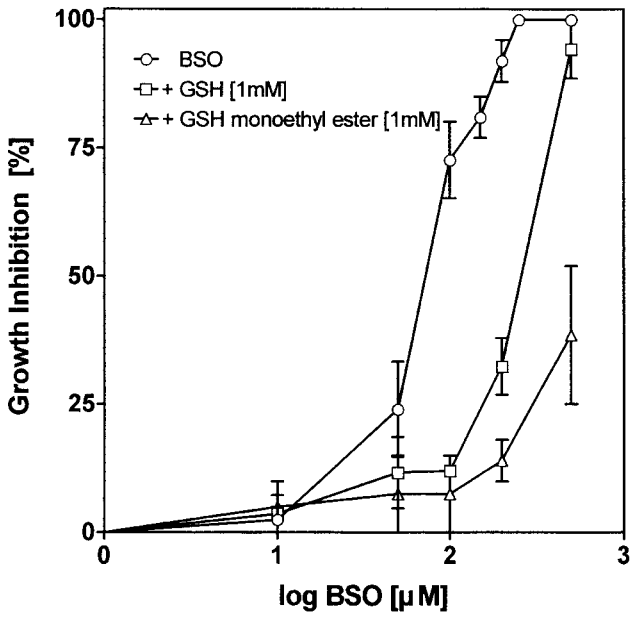

Figure 3 Effect of BSO alone, and in combination with GSH and GSH monoethyl ester respectively, on the proliferation of *P. falciparum* in *in vitro* culture

Drug screening was performed for 72 h as detailed in the Experimental section. Values are means ± S.D. for three separate experiments. The IC_{50} for BSO alone was determined to be 73 μM. The addition of 1 mM GSH increased the IC_{50} of BSO to 260 μM. The growth of *P. falciparum* was inhibited by less than 50 %, even in the presence of 500 μM BSO in combination with 1 mM GSH monoethyl ester.

In order to calculate the molarity of GSH in both compartments, the relative volume of each subunit (2/3 vol. by the host and 1/3 vol. by the parasite) was taken into account [35]. The GSH concentration in the RBC cytoplasm is 1 mM, which is a 50 % decrease of the GSH concentration found in non-infected RBCs. The GSH level in the parasite is even lower, namely 0.42 mM (Table 1).

Effect of BSO on the proliferation of *P. falciparum in vitro*

P. falciparum was cultured in the presence of increasing BSO concentrations for 24, 48 and 72 h. As shown in Figure 2, the proliferation of *P. falciparum* was completely inhibited by 200 μM BSO after 48 h of treatment. The IC_{50} of BSO was determined to be 73 μM.

Exogenously administered thiols in the culture medium led to an increase of the *in vitro* IC_{50} of BSO, whereby the supplementation of GSH monoethyl ester was more effective than that of GSH. The addition of 1 mM GSH increased the IC_{50} value of BSO four times. When 1 mM GSH monoethyl ester was added, even the highest BSO concentration (500 μM) inhibited parasite growth by less than 50 % (Figure 3).

Inhibition of γ-GCS activity and depletion of GSH in the parasite–host unit by BSO

BSO is a specific inhibitor of γ-GCS, the rate-limiting enzyme of GSH synthesis [21], and is known to deplete intracellular GSH levels [22,23]. Supplementation of non-lethal concentrations of

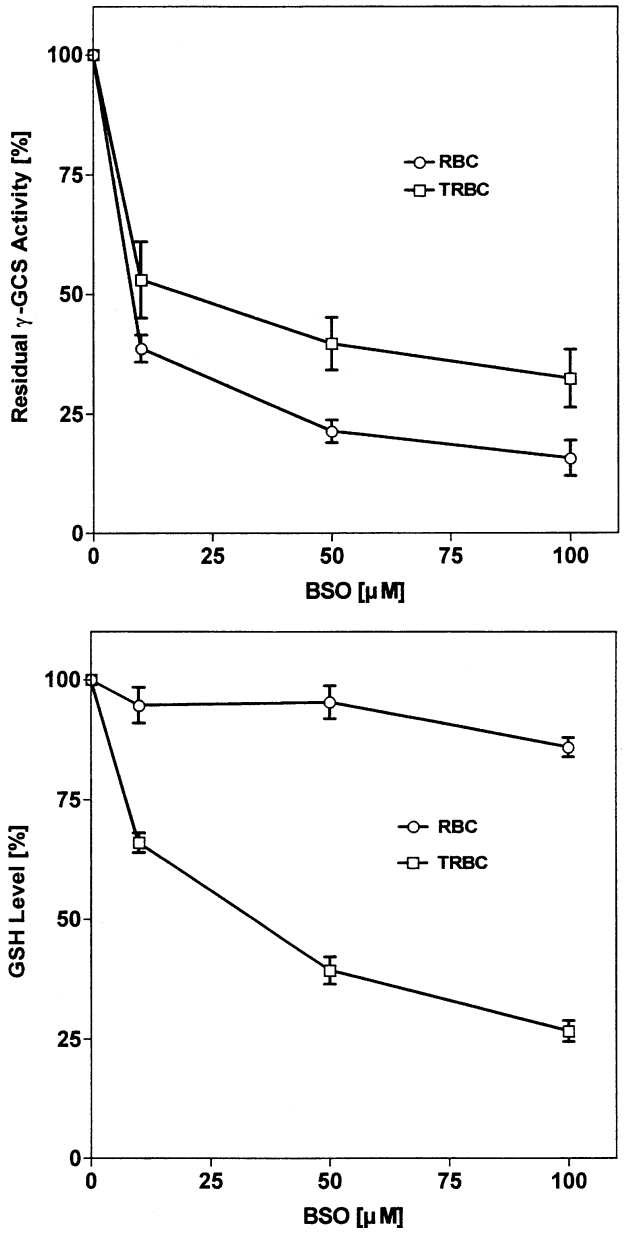

Figure 4 Effect of BSO on the γ-GCS activities and GSH levels in non-infected RBCs and TRBCs

The cells were incubated with different non-lethal concentrations of BSO (10–100 μM) for 24 h. TRBC were separated from non-infected RBCs by Gelafundin treatment and subsequent Percoll/alanine gradient. Results are given as means ± S.D. ($n = 3$). Upper panel: residual γ-GCS activities are compared with untreated controls in percentage. Lower panel: the corresponding residual GSH contents (percentage) are compared with control cells.

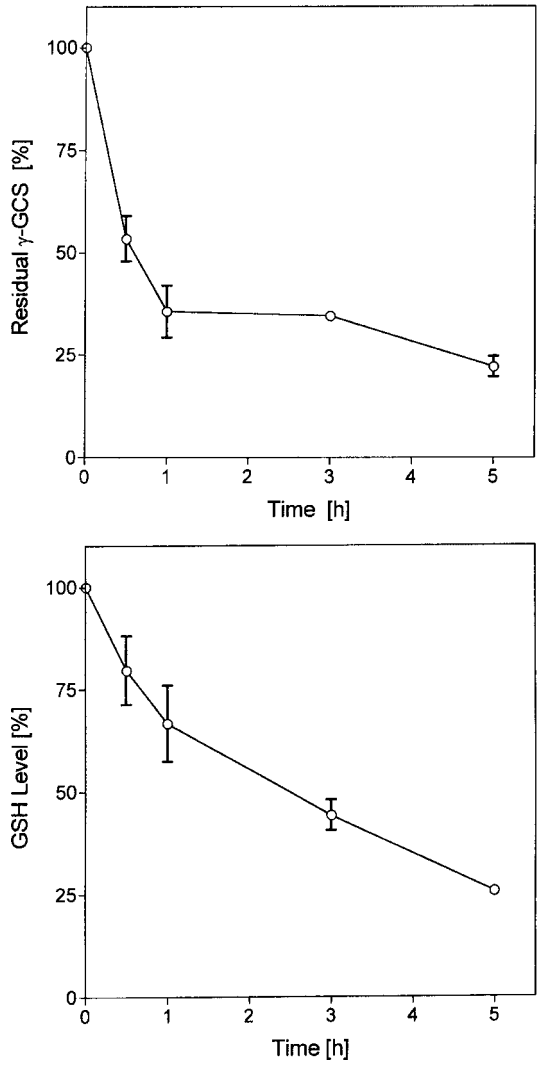

Figure 5 Short-term treatment of TRBCs with BSO

TRBCs were incubated with 100 μM BSO for up to 5 h. The Figure shows the relative residual γ-GCS activities (upper panel) and the corresponding relative GSH levels as a function of time (lower panel).

BSO (10–100 μM) for 24 h decreased the specific γ-GCS activities in non-infected RBCs (187.7 ± 20.8 nmol·min^{-1}·10^{10} cells^{-1}) as well as in TRBCs (141.6 ± 4.7 nmol·min^{-1}·10^{10} cells^{-1}) similarly (Figure 4, upper panel). In both cases the enzyme was inhibited in a dose-dependent manner. BSO at 100 μM led to 67 and 82% inhibition of the γ-GCS activities in TRBCs and non-infected RBCs respectively.

In non-infected RBCs the inhibition of γ-GCS activity by BSO had hardly any effect on the GSH levels (Figure 4, lower panel). BSO at 100 μM decreased the GSH level by 14%. In contrast, in TRBCs the reduced γ-GCS activity led to a considerable decrease of the GSH levels in a dose-dependent manner. BSO at 100 μM depletes the GSH level in TRBCs by 74%, suggesting a higher turnover rate of the tripeptide in the parasite–host unit.

To analyse the time-dependent effect of BSO on TRBCs, cells were exposed to 100 μM BSO for 0 h–5 h. The treatment resulted in a rapid decrease of the γ-GCS activity. After 3 h the enzyme was inhibited by about 70% (Table 1; Figure 5, upper panel). A higher degree of inhibition was not achieved by 100 μM BSO: even after 24 h of incubation a residual γ-GCS activity of 26% is detectable in TRBCs. The corresponding GSH levels showed a gradual decline (Figure 5, lower panel). The GSH content was decreased to less than 50% of the control value after 3 h of incubation. Taking into account that γ-GCS activity was not totally blocked, the TRBCs lost 50% of its GSH in considerably less than 3 h, which had to be replenished by the *de novo*-synthesis pathway in the untreated control.

In non-infected RBCs a 82% inhibition of the γ-GCS activity for 24 h had nearly no impact on the GSH level, which indicates

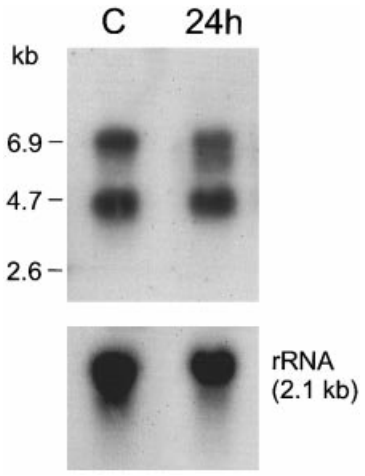

Figure 6 Northern-blot analysis of BSO-treated TRBCs

Total RNA from BSO-treated and untreated TRBCs was isolated using Trizol (Life Science) and separated on a formaldehyde/agarose gel. The RNA was transferred to a nylon membrane and probed with a radiolabelled γ-GCS probe overnight, washed and autoradiographed as described in [18]. Subsequently, the blot was re-probed with rRNA from *P. falciparum* as a loading control. Lane marked 'C', 20 μg of total RNA from untreated TRBCs; lane marked '24 h', 20 μg of total RNA from TRBCs treated with 100 μM BSO for 24 h. The size of the RNA markers (Boehringer-Mannheim) is given on the left in kb.

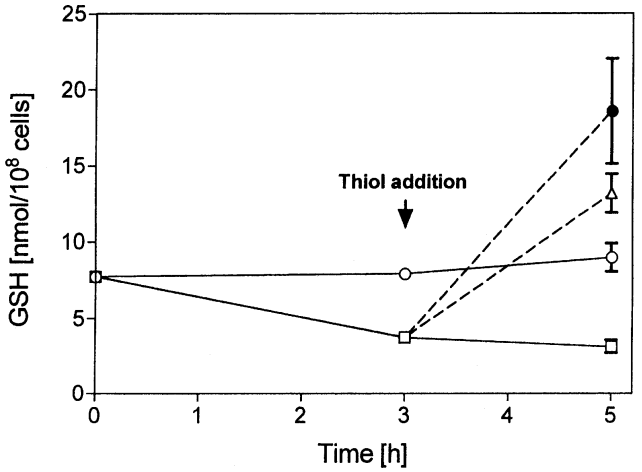

Figure 7 Effect of GSH and GSH monoethyl ester on the GSH level of BSO-treated TRBC

GSH levels were followed for 5 h. ○, □, GSH levels of untreated TRBC (○) and of TRBC treated with 100 μM BSO (□). 15 mM GSH (△) or 15 mM GSH monoethyl ester (●) were added to BSO-treated TRBC cells after 3 h as indicated by the arrow.

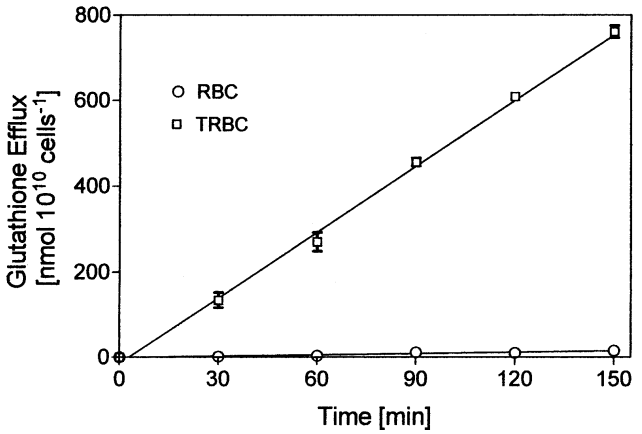

Figure 8 GSH efflux from *Plasmodium*-infected RBCs

Glutathione efflux was determined as described in the Experimental section.

an extremely low consumption of the tripeptide. Due to the infection with *P. falciparum* the requirement of GSH in the parasite–host unit was found to increase drastically. 50 % of the GSH in non-infected RBCs was calculated to be lost in 4–5 days [17,36]. TRBCs lose 50 % of their GSH in considerably less than 2.5 h.

Compartmental analysis was performed after TRBCs were treated with 100 μM BSO for 3 h. The γ-GCS activity and the GSH level of the host-cell cytoplasm, as well as of the parasite, were affected by the treatment similarly. The specific enzyme activity in the host (106.6 ± 20.8 nmol·min^{-1}·10^{10} cells^{-1}) and parasite (13.1 ± 2.6 nmol·min^{-1}·10^{10} cells^{-1}) compartments decreased to 26 and 22 % respectively. The GSH content in the red-blood-cell compartment decreases by 60 %, while the trophozoite contained 32 % of the GSH of the control (Table 1).

Northern-blot analysis of BSO-treated TRBC in comparison with untreated controls shows that the γ-GCS transciption level is not affected by drug treatment (Figure 6).

Effect of exogenously administered GSH and GSH monoethyl ester on the thiol level in TRBCs

Treatment with 100 μM BSO decreased the GSH level of TRBCs to 38 % within 3 h. The subsequent addition of 15 mM GSH or 15 mM GSH monoethyl ester to the medium replenished and increased the GSH contents of the TRBCs up to 1.31 ± 0.22 μmol·10^{10} cells^{-1} ($n = 3$) and 1.86 ± 0.48 μmol·10^{10} cells^{-1} ($n = 3$) respectively (Figure 7). The residual γ-GCS activity was not influenced by the thiols administered (results not shown). GSH monoethyl ester enters non-infected RBCs via diffusion, and a similar uptake is suggested for TRBCs.

The exogenously administered GSH was also taken up by TRBCs. In contrast, several studies showed that non-infected RBCs are not able to use extracellular GSH [17]. Thus the results presented here propose that the uptake of the tripeptide is mediated via a mechanism induced in the RBC membrane by the *Plasmodium* infection.

Efflux of GSH

The efflux of GSH from TRBCs was determined to be 306 ± 10 nmol·h^{-1} 10^{10}·cells^{-1} and thus is markedly higher than the efflux of GSH from non-infected RBCs (6 ± 1 nmol·h^{-1} 10^{10}·cells^{-1}) (Figure 8).

DISCUSSION

The thiol pattern of isolated *P. falciparum* was determined by separating monobromobimane derivatives by reversed-phase HPLC. Apart from GSH and cysteine, another peak was detected which could not be assigned to any of the standards used. In this way we excluded the possiblity that ovothiol A, which represents an abundant low-molecular-mass thiol in *Leishmania* [37], is present in the parasites.

The GSH metabolism of *P. falciparum*-infected RBCs has been of particular interest because the redox status was found to be an

important determinant for the survival of the malaria parasite [4,5]. Intracellular GSH levels depend on GR and on a functional *de novo* synthesis of the tripeptide. Inhibition of GR in *P. falciparum*-infected RBC by 1,3-bis-(2-chloroethyl)-1-nitrosourea results in a drastic decrease of the intracellular GSH level and has a plasmodicidal effect [12,13]. Recently, we have shown that the gene of *P. falciparum* γ-GCS is transcribed during the red-blood-cell cycle of the parasite [18]. To investigate the role of the GSH synthesis for the survival of the parasite in the host RBC, we have used BSO, a specific inhibitor of γ-GCS, in the present study.

Treatment with BSO has a lethal effect on *P. falciparum* in culture, with an IC_{50} of 73 μM. The drug concentration that completely inhibits the development of *P. falciparum* in culture was 200 μM. In phase I clinical trials in tumour therapy, steady-state plasma levels of 0.5–1.0 mM are reached with no or only very little toxicity to other tissues [23]. These data suggest that a drug concentration can be reached in the host which might be sufficiently high to selectively affect *P. falciparum* development. It was previously shown by Arrick et al. [24] that the administration of BSO cured *T. brucei*-infected mice as a result of GSH depletion in the parasites. However, the mice died, which was suggested to be a toxic reaction to the lysed trypanosomes, while BSO side-effects were excluded.

In order to examine the effect of BSO on γ-GCS activities and GSH levels in TRBCs and non-infected RBCs, we supplemented the culture medium with non-lethal drug concentrations for 24 h. Although γ-GCS activities decreased similarly in both cases, the depletion of GSH was much more pronounced in TRBCs, indicating a higher turnover of the tripeptide in the *P. falciparum*-infected cells.

Short-term treatment of TRBCs with BSO led to a rapid inhibition of γ-GCS activity. The corresponding GSH level decreased rapidly with a little delay. Compartmental analysis revealed that the γ-GCS activities and GSH content in host and parasite cells were equally affected. Although the γ-GCS was not totally blocked by the BSO concentration used, the GSH level decreased by more than 50 % after 3 h. Thus TRBCs had consumed more than 50 % of their GSH within this time, giving a $t_{\frac{1}{2}}$ for GSH turnover in TRBCs of considerably less than 2.5 h. One should bear in mind that 'turnover' here means a parameter that includes the efflux of GSH from the infected cell, the rate of oxidation of GSH to GSSG and utilization by the parasite. However, this turnover is most likely due to the oxidation of GSH to GSSG and its loss via an energy-dependent efflux mechanism. We have demonstrated, in accordance with Atamna and Ginsburg [38] and Ayi et al. [39], that the export of GSH is increased about 40-fold in TRBCs compared with non-infected RBC (306 $nmol \cdot h^{-1} \cdot 10^{10} cells^{-1}$ versus 6.0 $nmol \cdot h^{-1} \cdot 10^{10}$ $cells^{-1}$). Under conditions of oxidative stress this export rate can increase quite considerably in non-infected cells to about 65 $nmol \cdot h^{-1} \cdot ml^{-1}$ of cells [17] and is considered as a control for maintaining the intracellular redox status. Under normal conditions, non-infected RBC have a low GSH consumption with a half-life of the tripeptide of 4–5 days [17,36], and it has been suggested that the turnover rate of GSH depends mostly on its export rate.

In the present study the GSH level in RBCs parasitized by trophozoites of *P. falciparum* was determined to be reduced to 44 % of the value of non-infected RBC (Table 1), although the parasite is capable of its own GSH synthesis. Decreased GSH levels were also reported from *P. vivax*-infected RBCs [40]. In contrast, the amount of total GSH was found to be increased in RBCs infected with other *Plasmodium* species, such as *P. vinckei* and *P. berghei* [41,42]. The compartmental analysis of TRBCs (Table 1) shows that the GSH concentration in the residual erythrocytic cytoplasm (1 mM) was half of that of the uninfected RBC (2 mM). Although these results are in accordance with recently published data by Atamna and Ginsburg [38], the GSH concentration in the *P. falciparum* compartment was determined to be lower (0.4 mM compared with about 2.4 mM).

The plasmodicidal effect of BSO was distinct after 48 h (Figure 2). Parasite development is arrested in the trophozoite stage, and schizogony is prevented. These data are in accordance with our previous finding that γ-GCS is mainly transcribed in the trophozoite stage (24–30 h) in *P. falciparum*, where the formation of reactive oxygen species is increased as a result of haemoglobin digestion and thus the requirement for GSH is enhanced [18].

Considering the diverse functions of GSH in the cell, a multifactorial effect of GSH depletion on TRBCs is proposed. One of the major reasons certainly is the impairment of the antioxidative systems in the TRBCs by the observed GSH loss in both compartments. It has been shown numerous times that the disturbance of the delicate balance of pro- and anti-oxidants in *Plasmodium*-infected RBCs blocks parasite development [6–8,12,13].

Like TRBCs, melanoma cells are under an enhanced endogenous oxidative stress. Here, the major source of reactive oxygen species is melanin synthesis, in which a number of free-radical by-products are produced. It was proposed that GSH may be a critical factor in maintaining adequate reducing capacity in these tumour cells and that GSH depletion may be selectively toxic for them. A significant correlation between the melanin content of melanoma cell lines and their sensitivity to BSO was found [43].

In tumour cell lines, down-regulation of GSH levels by BSO is known to be an enhancer of cytotoxicity to several drugs. The ability to increase drug and pro-oxidant sensitivity by decreasing intracellular GSH has been demonstrated in a number of cell lines [22,23]. Recently, GSH depletion has been reported to sensitize resistant *P. berghei* and *P. falciparum* to chloroquine [44,45]. Therefore BSO needs to be tested in combination therapy with antimalarials and other drugs, especially with compounds that intensify the increased oxidative stress to which *P. falciparum* is exposed.

The addition of GSH monoethyl ester and GSH to the culture medium abolished the antimalarial effect of BSO (Figure 3). Therefore the alteration of the intracellular GSH level after supplementation with thiols was examined. Remarkably, both GSH monoethyl ester and GSH were taken up efficiently by BSO-treated TRBCs (Figure 7). GSH levels increased by 207 % and 146 % compared with untreated TRBCs, and 592 % and 419 % compared with the BSO-treated TRBCs respectively. These data indicate that the plasmodicidal property of BSO is attributable to the inhibition of γ-GCS and the subsequent depletion of intracellular GSH. Further, the dependence on a functional *de novo* synthesis of GSH for the survival of *P. falciparum* in the RBC is demonstrated.

GSH monoethyl ester is taken up by diffusion and is able to either restore or elevate GSH levels in several tissues and cell types, including RBCs [46]. A similar process is suggested here for TRBCs. The elevation of GSH levels produces increased resistance to several drugs, to radiation and to oxidative stress in different cell types [22]. Thus GSH monoethyl ester may be a tool for investigating drug resistance mechanisms in *P. falciparum*.

Interestingly, high concentations of GSH in the medium also restore the GSH level in TRBCs. Non-infected RBCs are not able to use exogenous GSH, since they are not supplied with the enzyme γ-glutamyltranspeptidase or a GSH transporter [17]. The infection with *P. falciparum* alters uptake of several

molecules, owing to the induction of new transporters and pores in the plasma membrane of the RBC [47]. Our data support the finding by Ginsburg and Atamna [38] that such induced permeability pathways are responsible for the uptake of the tripeptide GSH. Supplementation of 1 mM of either GSH or GSH monoethyl ester had no effect on the GSH level in the TRBCs (results not shown), but abolished the impact of BSO on the parasite survival. We suggest that, in this case, the added thiols entered the TRBCs at a lower rate than they were consumed and exported as GSSG. Obviously, higher concentrations of exogenous GSH or GSH monoethyl esters are necessary to establish an influence on the intracellular GSH level (Figure 7). The significance of this uptake is unclear, since the GSH concentration of less than 5 μM in the blood plasma is extremely low [48]. Remarkably, omission of GSH in the culture medium was reported to impair parasite development [49].

Owing to the drastic changes in GSH metabolism observed in *P. falciparum*-infected RBCs, the *de novo* synthesis of the tripeptide was found to be essential for the survival of the parasite. Therefore we propose γ-GCS as a potential target for chemotherapy of *P. falciparum*. Remarkably, the plasmodicidal effect of the inhibitor BSO is not due to its specificity towards its target enzyme in the parasite, but to the increased physiological requirement of the metabolite GSH in the infected RBCs.

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