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GLUTATHIONE REDUCTASE INHIBITORS AS POTENTIAL ANTIMALARIAL DRUGS

EFFECTS OF NITROSOUREAS ON *PLASMODIUM FALCIPARUM IN VITRO*

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Abstract—Malarial parasites are believed to be more susceptible to oxidative stress than their hosts. BCNU(1,3-bis(2-chloroethyl)-1-nitrosourea) and HeCNU(1-(2-chloroethyl)-3-(2-hydroxythyl)-1-nitrosourea), inhibitors of the antioxidant enzyme glutathione reductase, were found to prevent the growth of *Plasmodium falciparum* in all intraerythrocytic stages. When exposing infected red blood cells to 38 μM BCNU or 62 μM HeCNU for one life cycle of synchronously growing parasites, the parasitemia decreased by 90%. During the formation of new ring forms, the parasites are even more susceptible to these drugs. The treatment with BCNU or HeCNU produced a rapid depletion of GSH in the parasites and their host cells; in addition, protection against lipid peroxidation was impaired in these cells. Possible mechanisms for the antimalarial action of the inhibitors are discussed. Our results suggest that erythrocyte glutathione reductase, an enzyme of known structure, might be considered as a target for the design of antimalarial drugs.

Oxidative stress or oxidant stress in biomedical systems denotes a disturbance of the prooxidant-antioxidant balance in favour of the former [1]. Clinical observations and experimental evidence suggest that oxidative stress plays a dominant role in the defense against parasitic infections [2–5]. As a point in case, inborn and acquired conditions leading to intracellular oxidant stress protect, although not absolutely, red blood cells from the malaria parasite *Plasmodium falciparum* [2–4, 6]. This is in keeping with the observation that parasitized erythrocytes are more susceptible to the toxic effects of H_2O_2 than normal red cells [7]. A major defense system for the detoxification of reactive oxygen species in red blood cells is the glutathione redox cycle containing the two enzymes glutathione peroxidase ($ROOH + 2\,GSH \rightarrow ROH + H_2O + GSSG$) and glutathione reductase ($GSSG + NADPH + H^+ \rightarrow 2\,GSH + NADP^+$) [8]. Consequently it is to be expected that inhibitors of these enzymes impair the intraerythrocytic growth of malaria parasites. Here we describe the antimalarial effects of the glutathione reductase inhibitors BCNU‡ [8–11] and HeCNU [3, 12, 13]. BCNU is widely used in tumour therapy [9, 10, 14]; HeCNU, a less toxic derivative of BCNU, is of interest because it was found to cure rodent malaria caused by *Plasmodium vinckei* [15].

MATERIALS AND METHODS

Materials. All reagents (from Boehringer, Mannheim, Merck, Darmstadt, Roth, Serva, Heidelberg or Sigma, St. Louis, MO) were of the highest purity available. RPMI-1640 medium was obtained from Biochrom (Berlin). BCNU and HeCNU were kind gifts of Professor G. Eisenbrand, Kaiserslautern University.

Cultivation and isolation of the parasites. P. falciparum (FCB strain) was cultured according to the candle jar method [16] in RPMI-1640 medium supplemented with 25 mM HEPES, 32 mM $NaHCO_3$, 50 mg/ml hypoxanthine, 100 mg/l gentamycine and 10% human serum from A(+) blood. The pH of the culture medium was 7.4 at 37°. In this paper the supplemented medium is designated RPMI medium; the supplemented medium plus serum is called culture medium. At about 15% parasitemia with the parasites being present as trophozoites and schizonts, the erythrocytes were collected by centrifugation and washed with isotonic medium (68 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 5 mM glucose in 50 mM sodium phosphate, pH 7.4). The cells were lysed by incubation at 37° for 10 min in 20 vol. of 150 mg/l saponin in isotonic medium [17]. The parasites were pelletted by centrifugation at 500 *g*, washed twice with isotonic medium and used immediately.

Concentration effect of BCNU and HeCNU on the growth of P. falciparum *in vitro.* Sorbitol-synchronized parasites [18] were used in all experiments; the initial parasitemia was 1.5% with the parasites being in the ring stage. Infected erythrocytes (25 μl

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‡ Abbreviations: BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; HeCNU, 1-(2-chloroethyl)-3-(2-hydroxyethyl)-1-nitrosourea; GR, glutathione reductase (EC 1.6.4.2); *t*-BHP, *t*-butyl hydroperoxide; TBA, thiobarbituric acid.

in 500 μl medium) were cultured in 24-well plastic plates (Greiner, F.R.G.) and exposed to various concentrations of BCNU or HeCNU. Final parasitemia was determined after 48 hr of culture by counting the number of parasites per 2000 erythrocytes in Giemsa-stained smears; the average was calculated from duplicate culture dishes [19].

Effect of BCNU on erythrocyte invasion and the formation of new rings. Highly synchronous parasites obtained by treatment with 5% sorbitol with a cultural interval of 30 hr were grown in 24-well plastic plates. The cultures were divided into three groups, each group comprising 5 wells.

Group 1.

Infected erythrocytes (10% parasitemia) were exposed to BCNU from 14 to 22 hr after the second synchronization. Then, the erythrocytes were washed three times and added to washed fresh erythrocytes to obtain 1.2% parasitemia. The culture was then continued in BCNU-free medium until 52 hr.

Group 2.

Infected erythrocytes with 1.2% parasitemia were cultured in BCNU-free medium until 44 hr and then exposed to BCNU for 8 hr.

Group 3.

The parasites in the control group were cultured without inhibitor. Final parasitemia was monitored for the three groups by microscopic examination at 52 hr. Statistical analyses were performed by Student's two-tailed test for unpaired data. The results are expressed as means ± standard error of five test samples.

Glutathione reductase assay and protein determination. Isolated *P. falciparum* and washed erythrocytes were dissolved with 4 vol. of solution T (10 mM $MgCl_2$, 10 mM mercaptoethanol, 4 mM EDTA, 4% Triton X-100, 50% glycerol in 10 mM potassium phosphate, pH 6). Glutathione reductase was assayed according to Krohne-Ehrich *et al.* [20]. The enzyme activity is given in U/ml cells or in mU/mg protein. Protein was determined as described by Heinzel *et al.* [21].

Effect of BCNU and HeCNU on the glutathione levels of host cells and parasites. Five percent suspensions of washed fresh erythrocytes and infected erythrocytes in RPMI-medium were exposed at 37° to 500 μM BCNU or 500 μM HeCNU. Aliquots containing 20 μl erythrocytes were taken at different times and prepared for the determination of glutathione according to the method of Anderson [22].

Effect of BCNU on t-*butyl hydroperoxide-induced lipid peroxidation.* A 5% suspension of erythrocytes in RPMI-medium was incubated with 1 mM *t*-BHP and 0, 100 or 500 μM BCNU, respectively. Lipid peroxidation was measured according to Yagi's method [23] with modifications: 30 μl erythrocytes was pipetted into 2.5 ml distilled water and 0.5 ml TBA reagent (equal volumes of 0.67% thiobarbituric acid and acetic acid) was added to the solution. The reaction mixture was heated for 60 min at 95° in a water bath. After cooling, 0.5 ml 10% phosphotungstic acid was added, followed by 3 ml chloroform in order to remove dispersed lipids. After shaking the sample for 3 min, the two phases were separated by centrifugation. The water layer was taken for fluorometric measurement at 553 nm with excitation at 515 nm. The results are expressed as equivalents of lipid peroxidation (nmol TBA/g Hb).

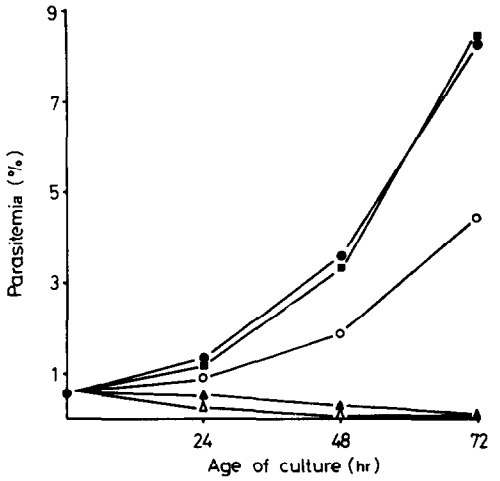

Fig. 1. Inhibition of parasite growth by BCNU and the effect of glutathione. *P. falciparum* was cultured in medium without additions (●), in medium with 20 mM GSH (■), with 20 mM GSSG (○), with 100 μM BCNU (△) and with 100 μM BCNU plus 20 mM GSH (▲).

RESULTS

General observations

The capacity of glutathione reductase in human erythrocytes is 1.0–1.4 U/ml cells corresponding to 3.5–5 U per g haemoglobin; a similar activity of the enzyme was determined for *P. falciparum* (4–4.8 U/g protein). The activity of red blood cell GR was found to decrease by less than 20% in 3 days under conditions used for culturing the intraerythrocytic forms of *P. falciparum*.

BCNU and HeCNU led to complete (≥98%) and practically irreversible inhibition of intracellular glutathione reductase. Haemolysis was not observed which is consistent with the finding of Loos *et al.* [24] that erythrocytes without detectable GR activity can fulfil their functions *in vivo*.

Inhibition of parasite growth by BCNU and HeCNU in vitro

As shown in Figs 1 and 2 the growth of *P. falciparum* is completely inhibited by 100 μM BCNU or 100 μM HeCNU, even in the presence of a high GSH level in the culture medium. The concentration-dependence of the inhibitory effects is shown in Fig. 3. When exposing the parasites for one life cycle to the nitrosourea compounds, IC_{50} and IC_{90} were found to be 13 μM and 38 μM for BCNU; the corresponding values for HeCNU were 18 μM and 62 μM.

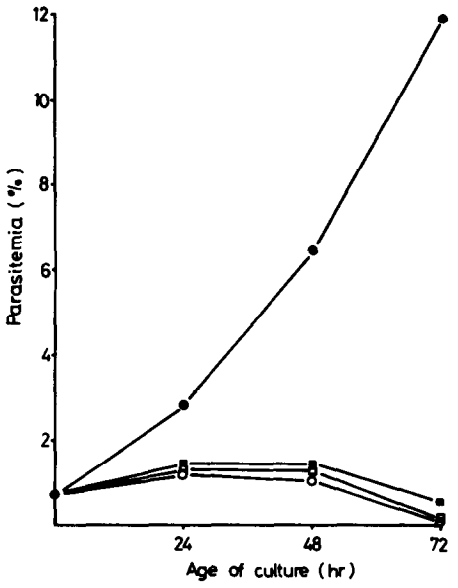

Fig. 2. Inhibition of parasite growth by HeCNU. *P. falciparum* was cultured in medium without additions (●), medium with 100 μM HeCNU (○), with 100 μM HeCNU plus 5 mM GSH (□) and with 100 μM HeCNU plus 20 mM GSH (■). Each point represents the mean of three experiments.

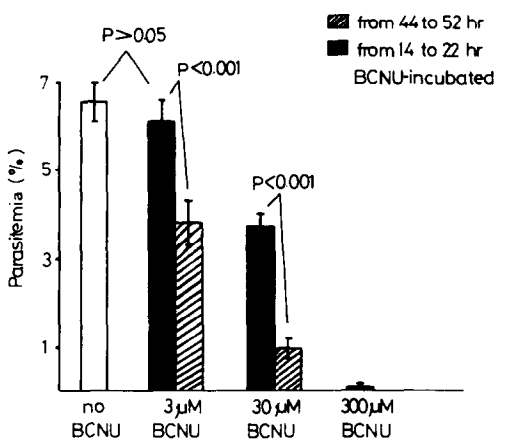

Fig. 4. BCNU effect on parasite reinvasion and the formation of new rings. Highly synchronous parasites were exposed to BCNU for 8 hr during the late ring stage (14–22 hr) and during the formation of new rings (44–52 hr). Final parasitemia was determined at 52 hr. The results are expressed as means ± standard error of five samples.

Sensitivity of the parasites to BCNU and HeCNU at different stages of their life cycle

The inhibitory effects of BCNU and HeCNU on parasite growth are stage-independent. This was shown by exposing synchronous cultures of *P. falciparum* to the inhibitors either during the ring stage (from 0 to 24 hr of the intraerythrocytic life cycle) or during the trophozoite/schizont stage lasting from 28 to 48 hr (Fig. 3). The transition from one life cycle to another which includes merozoite release from a parasitized red blood cell, invasion of new erythrocytes and ring formation of the parasites, comprises the time interval from about 44 to 52 hr. During these 8 hours the parasites were found to be more susceptible to BCNU than at other stages (Fig. 4). At a concentration of 3 μM, BCNU inhibits the formation of new rings ($P < 0.001$) but has no statistically significant effect ($P > 0.05$) on the growth and differentiation of the ring forms present at 14–22 hr of a cycle.

Depletion of glutathione in the parasites and their host cells

In many cells the inhibition of glutathione reductase leads to a decrease of the GSH level [10, 11, 25, 26]. As shown in Fig. 5 this is also the case for erythrocytes and intraerythrocytic *P. falciparum*. In the presence of 500 μM BCNU the intra-

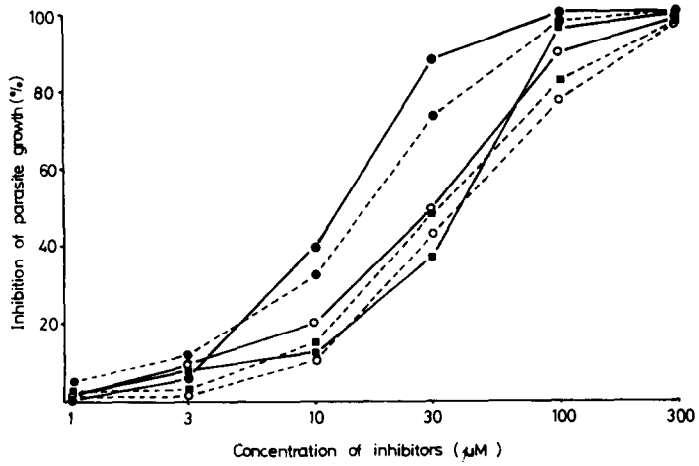

Fig. 3. Concentration effect of BCNU and HeCNU on the inhibition of *P. falciparum*. Synchronous *P. falciparum* was cultured in the medium with various concentrations of BCNU (——) or HeCNU (---) for a whole life cycle (from 0 to 48 hr) (●), during the ring stage (0–24 hr) (■), or the trophozoite/schizont stage (28–48 hr) (○).

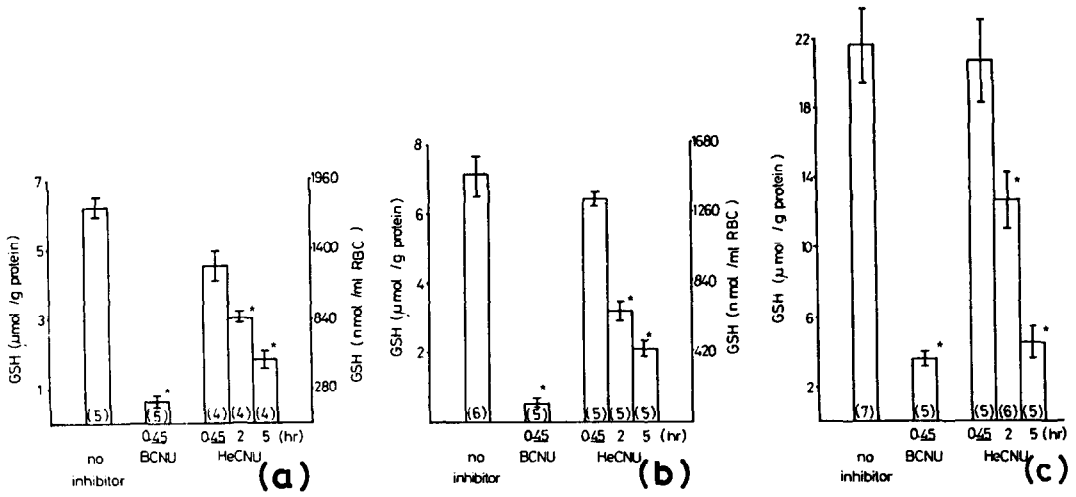

Fig. 5. Depletion of glutathione by BCNU and HeCNU. The cells were incubated in RPMI medium with 500 µM BCNU or 500 µM HeCNU. GSH was determined at 45 min, 2 and 5 hr. Values are means ± standard error. Numbers within the bars indicate the numbers of samples. * $P < 0.001$. (a) erythrocytes; (b) infected erythrocytes with 15% parasitemia; (c) *P. falciparum* isolated from parasitized red cells.

cellular GSH concentration drops to 10% of the initial value within 45 min; HeCNU has a similar effect but it acts more slowly than BCNU (Fig. 5). The GSH level in the erythrocyte and the GSH level in the parasite are affected by the glutathione reductase inhibitors in a similar fashion, the time courses and the resulting GSH levels being almost identical for the two cells (data not shown).

Enhanced lipid peroxidation of host erythrocytes in the presence of BCNU

Glutathione and glutathione reductase are parts of the glutathione redox cycle which protects cells against damage by oxidants [1, 8, 10]. Consequently it is to be expected for situations of oxidative stress that the presence of glutathione reductase inhibitors leads to increased peroxidation of membrane lipids and other cell constituents. Figure 6 shows that red cells are susceptible to 1 mM *t*-butyl hydroperoxide and that the effect of this oxidative stressor is clearly enhanced by BCNU in a concentration-dependent fashion.

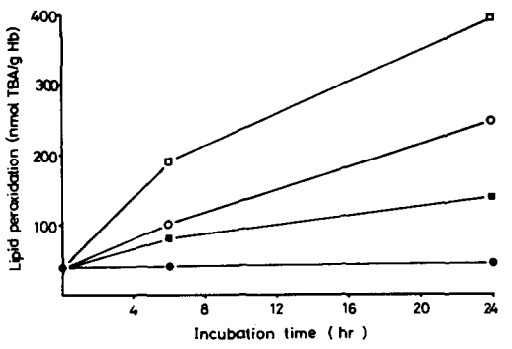

Fig. 6. Lipid peroxidation of erythrocytes in the presence of BCNU. Erythrocytes were incubated in RPMI-medium without additions (●), in medium with 1 mM *t*-BHP (■), with 1 mM *t*-BHP plus 100 µM BCNU (○) and with 1 mM *t*-BHP plus 500 µM BCNU (□).

DISCUSSION

The results reported here indicate that inhibitors of glutathione reductase are active as antimalarial agents *in vitro* (Figs 1 and 2). The different intra-erythrocytic stages are equally sensitive to these drugs (Fig. 3), but in the events leading to merozoite invasion and ring formation the parasite is more susceptible to BCNU (Fig. 4). This is in keeping with the observation of Wozencraft *et al.* [27, 28], that merozoite invasion is precluded when the parasites are exposed to an oxidant-generating system.

BCNU and HeCNU cause a drop of the glutathione levels to 10% in the parasites and their host cells within 45 min (Fig. 5). The most likely explanation for the loss of glutathione is as follows. The normal cell metabolism leads to the oxidation of GSH to GSSG. In the absence of glutathione reductase activity GSSG is not re-reduced but exported from the cells instead. Such an oxidation-excretion mechanism for glutathione has indeed been demonstrated for human erythrocytes [29]. In our experiment the *de novo* synthesis of reduced glutathione (GSH) apparently did not keep up with the loss, although the RPMI medium contains the constituent amino acids of GSH.

An obvious question is whether it is the loss of glutathione or the inactivation of glutathione reductase which is the more important for the inhibition of parasite growth. The two mechanisms cannot be separated sharply from each other because the depletion of glutathione is accompanied by a low [GSH]/[GSSG] ratio which probably leads to inhibition of protein synthesis and DNA synthesis in the parasite [8].

Depletion of glutathione

According to literature reports a low level of GSH

in host erythrocytes interferes with the growth of *P. falciparum* [30, 31]. This is consistent with studies on red blood cells whose glutathione reductase had been inactivated by BCNU with a remaining activity of less than 2%. In a BCNU-free culture medium these cells did not serve as host cells for *P. falciparum* but when GSH (20 mM) was added to the medium parasite growth was found to be normal [11]. As shown in Fig. 1, parasites do not grow when *both* GSH and BCNU are present in the culture medium. These findings together suggest that a high GSH level is essential for the invasion process and/or for the early development of *P. falciparum*. As soon as the parasite contains sufficient glutathione reductase of its own [32–34] it can grow unless the newly-synthesized enzyme is inhibited by BCNU.

Inactivation of glutathione reductase

The inactivation of the enzyme could play the predominant role when parasitized red blood cells are exposed to oxidative stress. Oxidative stress is believed to be a major defense mechanism against malarial parasites [2, 3, 7, 35]. As shown in Fig. 6, BCNU impairs the protection of host erythrocytes against oxidative damage caused experimentally by *t*-butyl hydroperoxide. This is to be expected because peroxides are detoxified by the glutathione redox cycle containing glutathione peroxidase and glutathione reductase as enzymes [8, 33, 36].

Glutathione reductase is not essential for the normal function of erythrocytes [9, 24]. This fact is important when considering glutathione reductase inhibitors for the prevention and treatment of malaria. In addition, a single dose of the inhibitor is expected to have a longer-lasting effect in erythrocytes than in cells which are able to synthesize the enzyme *de novo*. Lens fibres which like erythrocytes cannot synthesize proteins are not expected to be reached by lipophilic compounds such as BCNU. BCNU and even HeCNU which is curative against *Plasmodium vinckei* infections in mice [15] might be too toxic to be considered as antimalarial agents. It remains to be studied whether the toxic side-effects are due to the inhibition of glutathione reductase. If not, glutathione reductase, an enzyme of known three-dimensional structure [37] and known stereochemistry of catalysis [38], might be considered as a target for drug design. A number of compounds with very different structures, such as HeCNU, paraquat and triiodothyronine are known as ligands of the enzyme [8] and can serve as starting points for tailoring drugs. As a first step towards designing better inhibitors, crystals of BCNU and HeCNU-inactivated glutathione reductase have recently been analyzed by X-ray crystallography [39].

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