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OXIDATIVE STRESS AS A DEFENSE MECHANISM AGAINST PARASITIC INFECTIONS

R.H. SCHIRMER,† T. SCHÖLLHAMMER, G. EISENBRAND and R.L. KRAUTH-SIEGEL

Institut für Biochemie II der Universität, 6900 Heidelberg, and Institut für Lebensmittelchemie und Toxikologie der Universität, 6750 Kaiserslautern, FRG

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Many parasites — including the causative agents of malaria, Chagas' disease and schistosomiasis — are more susceptible to reactive oxygen species (ROS) than their hosts are. This is manifested by one or more of the following criteria: 1. Susceptibility of the parasite to ROS *in vitro*; 2. macrophage-based defense mechanisms against the parasite *in vivo*; 3. successful therapy using agents which lead to oxidative stress; 4. selection advantage (with respect to parasite infections) of human populations whose antioxidant capacity is impaired by a gene defect or by strong oxidants in their staple food.

Our laboratory is involved in developing inhibitors against antioxidant enzymes thus mimicking natural experiments. Since glutathione reductase is a protein of known atomic structure the methods of drug design by receptor fit (DDRF) can be applied for this enzyme. Another promising target enzyme is trypanothione reductase which was found so far only in trypanosomatids, and specifically, not in their hosts. Consequently the trypanothione pathway may be a general target in the design of drugs against diseases caused by trypanosomes and leishmanias.

KEY WORDS: Malaria, trypanosomal diseases, glutathione reductase, HeCNU, trypanothione reductase, drug design.

ABBREVIATIONS: BCNU, 1,3-*bis* (2-chloroethyl)-1-nitrosourea; DTE, dithioerythritol; GR, glutathione reductase; HeCNU, 1-(2-chloroethyl)-3-(2-hydroxyethyl)-1-nitrosourea; HPLC, high-performance liquid chromatography; SDS, sodium dodecylsulfate; TFA, trifluoroacetic acid; TR, trypanothione reductase; *Tris*, *tris*(hydroxymethyl)-aminomethane; TS_2, trypanothione disulfide; $T(SH_2)$, dihydrotrypanothione.

INTRODUCTION

Clinical observations and experimental evidence suggest that oxidative stress[1] plays a dominant role in the host's defense against parasitic infections. Diseases which have been studied under this aspect include malaria,[2–4] sleeping sickness and other trypanosomal diseases,[5–7] kala azar,[8,9] and schistosomiasis.[10,11] On the basis of these studies it has been argued that unicellular parasites rather than tumour cells represent the selecting force which shaped the ROS-producing cytotoxic cells of the immune system during evolution.[12] This concept might turn out to be fruitful for clinical research because it stresses the similarities between malignant tumours and poorly adapted parasites from a new perspective.

The present article reviews the evidence for the role of ROS in parasitic diseases using malaria and trypanosomiasis as examples. It includes therapeutic measures,

† To whom correspondence should be sent.

vaccination and chemotherapy, which expose parasites or parasitized host cells to increased oxidative stress. As an example of active drug research we report on the modification of the antioxidant enzyme glutathione reductase by HeCNU. This compound is used clinically in tumour therapy and experimentally in malaria research.[13,14]

MATERIALS AND METHODS

Glutathione reductase ($NADPH + GSSG + H^+ \rightarrow NADP^+ + 2\,GSH$; EC 1.6.4.2) from human red blood cells was prepared and assayed according to Krohne-Ehrich *et al.*[15]

HeCNU was synthesized as described.[13] 25 mg (= 130 μmol) 1-(2-^{14}C-chloroethyl)-3-(2-hydroxyethyl)-1-nitrosourea (^{14}C-HeCNU) with a specific radioactivity of 36 700 Bq/μmol was a kind gift from ASTA Pharmaceuticals.

The reference compound S-(2-hydroxyethyl)-L-cysteine was kindly provided by Dr. W. Stahl, Kaiserslautern. The PTH-derivative of S-(2-hydroxyethyl)-L-cysteine was prepared according to published procedures.[16]

Inhibition of the Enzyme by ^{14}C-HeCNU

Stock solutions of 300 mM NADH in 100 mM phosphate, 200 mM KCl, 1 mM EDTA, adjusted to pH 7.4 at 25°C with KOH (buffer P) and of 250 mM ^{14}C-HeCNU in ethanol with a specific radioactivity of 37 Bq/nmol, were prepared. 52.4 mg (= 1 μmol subunit) glutathione reductase was dialyzed in a small volume against buffer A and then incubated with ^{14}C-HeCNU in the presence of NADH as a reductant. The reaction mixture contained 400 μM GR, 10 mM NADH and 10 mM ^{14}C-HeCNU in a total volume of 2.5 ml buffer P. After 24 h at 37°C the same amounts of NADH and ^{14}C-HeCNU were added and the reaction was allowed to proceed for a further 22 h at 37°C, until $\geqslant 90\%$ of the enzyme activity was inhibited. The reaction was stopped by dialyzing against buffer P and then against 500 mM *Tris*·acetate, pH 8.7 at 4°C. The resulting derivative of glutathione reductase contained 52.5 Bq/nmol, corresponding to 1.4 nmol ^{14}C per nmol enzyme subunit.

Carboxymethylation of the ^{14}C-labelled Enzyme

^{14}C-labelled GR was percarboxymethylated using iodoacetate under denaturing and reducing conditions.[17,18] 10 ml reaction mixture contained 100 μM enzyme subunit, 450 μM DTE, 6 M guanidinium chloride and 5.4 mM iodoacetate in 500 mM *Tris*·acetate, pH 8.7. After 1 h incubation at 25°C in the dark, the alkylation of the enzyme was stopped by the addition of DTE to a final concentration of 53 mM. Then the sample was dialyzed 3 times against 10 mM acetic acid and subsequently against 200 mM N-ethylmorpholinium·acetate, pH 8.1, at 4°C.

Tryptic Digestion

The percarboxymethylated enzyme derivative was cleaved by trypsin. 1 μmol enzyme was incubated with 2.6 mg trypsin in 20 ml 200 mM N-ethylmorpholinium·acetate, pH 8.1, containing 0.05% SDS for 1 h at 37°C. The protein:protease ratio was 20:1 (w:w). Digestion was stopped by lyophilization.

Identification of the ^{14}C-labelled Amino Acid Residue

The lyophilized tryptic digest was dissolved in 1 ml 0.1% TFA. Aliquots thereof were separated by reversed-phase high-performance liquid chromatography (HPLC) on a Lichrosorb RP 18–7 μ column using a linear 0–60% gradient of 0.1% trifluoroacetic acid as buffer A and 0.1% trifluoroacetic acid with 50% CH_3CN as buffer B during 60 min at a flow rate of 1 ml/min.[19] Fractions of 0.5 ml were collected. Only two radioactive peaks were eluted from the column with 18% buffer B (peak I) and 46% buffer B (peak II), respectively. Fraction I contained 33%, fraction II 67% of the total radioactivity.

The radioactive peptides were purified further on a Pharmacia Pep RPC column with a linear gradient of 0–45% B during 90 min. Fraction I was now eluted with 7% buffer B; according to amino acid analysis it contained the N-terminal tryptic peptide t(1–3) of the enzyme; pool II was separated into 4 peaks; one of them eluted with 31% buffer B, was radioactive and found to contain the active site peptide t(54–66). 300 pmol of peptide t(54–66) was subjected to automated Edman degradation in a non-commercial gas phase sequenator.[20] The sequence analysis, kindly performed by Dr. Rainer Frank, EMBL, Heidelberg, revealed that peptide t(54–66) contained 2-hydroxyethylcysteine in position 58. Amino acid analyses using o-phthalaldehyde derivatisation confirmed the presence of 2-hydroxyethylcysteine in both peptides t(1–3) and t(54–66), respectively.

RESULTS AND DISCUSSION

When dealing with oxidative stress as a defense mechanism against a parasitic disease, four points should be considered:

1) Susceptibility of the parasites to ROS *in vitro*;

2) macrophage-based mechanisms against the parasite *in vivo*;

3) selection advantage of human populations whose antioxidant capacity is impaired by a gene defect and/or by the presence of strong oxidants in their staple food;

4) chemotherapy with compounds which act as oxidative stressors.

These four criteria are to be discussed for the case of malaria, a parasitic disease of vertebrates. Since 1975 the most dangerous form of this disease, malaria tropica of man, is spreading again and at a phantastic rate; according to the WHO[21] it has regained its classical role as one of the greatest natural threats to human health and human welfare. Malaria parasites (*Plasmodium species*) undergo a complicated life cycle but the actual disease is caused by the multiplication of the parasite in red blood cells. A parasite enters a red blood cell and multiplies 8 to 16 fold within 2 days. Then the host cell ruptures and new red blood cells are invaded. The release of the parasites leads to an irregular fever which costs the patient's energy household up to 5000 kcal per day.[22] Consequently it is often difficult to decide whether the patient, as a rule a child under 5 years of age, dies of malaria, of acute starvation or of an intercurrent disease.

Susceptibility of the Parasites to ROS in vitro

The findings presented here often refer to a given unit of a specific malaria parasite and its specific mammalian host but they are probably valid for most types of malaria.

In vitro, parasitized red blood cells are killed by H_2O_2 at a concentration of 100 μM, whereas non-parasitized erythrocytes tolerate this concentration.[3] In this context one should remember that the H_2O_2 concentration in the contact zone between macrophages and tumour cells has been estimated to reach values of up to 10 mM.[23,24]

The presence of antioxidant systems within the red cell itself is clearly a barrier to parasite killing by ROS, and it is significant that parasites in mature red cells are much more susceptible than the same species of parasite in reticulocytes or young red cells which have higher levels of catalase and other antioxidant enzymes. It is believed that the switch of the parasite from adult red cells to reticulocytes as host cells is due to the production of ROS by macrophages. Since parasites invading mature red cells are potentially more rapidly fatal, any response that leads to ROS release in the right microenvironment could well be life-saving.[3]

Results from Dr. Jung's laboratory indicate why H_2O_2 is so toxic for malaria parasites.[25] It was found that the glutathione peroxidase of the parasite differs from that of the red blood cell. The parasite's enzyme does not contain selenium and it is different in its substrate specificity: Although it easily reduces organic hydroperoxides like tertiary butyl hydroperoxide and cumene hydroperoxide it is unable to reduce H_2O_2.

Macrophage-based Defense Mechanisms of the Host

The involvement of macrophages in malaria has been reviewed recently.[2-4] Apart from ROS, tumour necrosis factor and possibly other macrophage products are parasiticidal. Spleen and liver macrophages are both more numerous and more activated during malaria as judged by ROS production and direct cytotoxicity towards the L929 cell line. When different malaria forms were compared, a striking hierarchy emerged: the less severe the infection the more activated were the macrophages.[3] Of practical importance are the findings that γ-interferon (IFNγ),[26] but also protective antibodies,[27] are potent inducers of parasite killing by macrophages.

Selection Advantage of Human Populations with Impaired Antioxidant Capacity

Five main genetically controlled traits in man are common in populations with a history of exposure to malaria tropica and rare elsewhere: Sickle-cell anaemia, thalassaemia, haemoglobin E (HbE), persistent fetal haemoglobin (HbF) and glucose-6-phosphate dehydrogenase (G6PDH) deficiency. These mutations appear to share the functional defect of exposing the red cell to higher than normal intraerythrocytic oxidative stress.[28]

The combined effect of a genetic defect and the dietary habits of a population have been studied in great detail for the case of favism. Favism is a hemolytic anaemia caused by glucose-6-phosphate-dehydrogenase deficiency but precipitated by the ingestion of fava beans and probably another so far unknown factor.

Intuitively one would expect that in Mediterranian and Middle Eastern countries the high consumption of fava beans – a major protein source – should have selected *against* G6PDH-deficiency. In actual fact, as noted by Huheey and Martin,[29] the frequency of mutated G6PDH genes is highest in these countries. This apparent contradiction was resolved by Golenser *et al.* who showed that fava beans contain compounds with antimalarial activity which are especially effective in red cells of deficient individuals. Thus – as stated by Clark *et al.*[2,28] – "antimalarial drugs acting

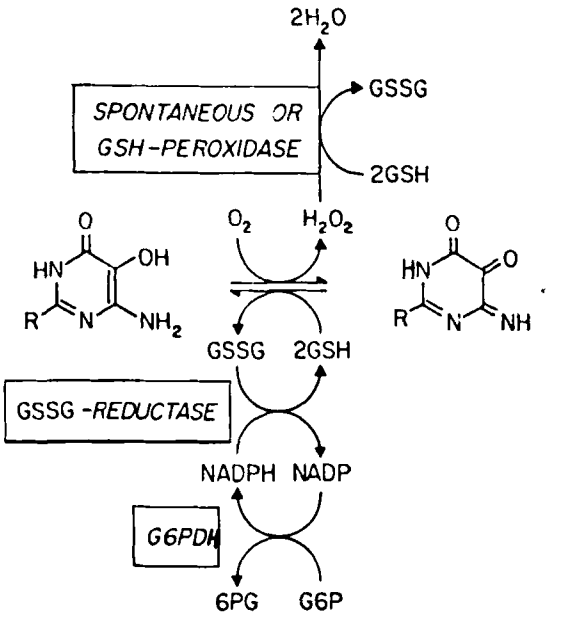

FIGURE 1 *Favism: The action of broad bean pyrimidines in the red blood cell.*[31] One of the 2e-redox-cycling pyrimidines is isouramil (R ≙ OH). When isouramil reacts with O_2, H_2O_2 representing oxidant stress is formed. Both H_2O_2 and oxidized isouramil are reduced at the expense of (four) GSH molecules. The oxidized glutathione is reduced by the flavoenzyme glutathione reductase. G6PDH, glucose-6-phosphate dehydrogenase; 6PG, 6-phosphogluconolactone; G6P, glucose 6-phosphate.

through oxidative stress may have been in the basic human diet in some regions for many centuries, and indeed helped shape the genetic pool in those populations". Results with *P. vinckei*-infected mice show that even in animals with normal red cell biochemistry appropriate doses of these compounds can be lethal to malaria parasites *in vivo*.[2]

Chemotherapy with Compounds Which Act as Oxidative Stressors

The biochemistry of favism is summarized in Fig. 1. This scheme and the observation that favism occurred with a patient showing GR deficiency[32] instead of G6PDH deficiency suggested to us that the biochemistry of favism might be mimicked by inhibiting the enzyme glutathione reductase in human erythrocytes (Fig. 2). The three-dimensional structure of this flavoenzyme which catalyzes the reaction $NADPH + GSSG + H^+ \rightarrow NADP^+ + 2\,GSH$ is known,[33,34] so that the interactions of glutathione reductase and inhibitors can be studied in atomic detail[35] and the design of new inhibitors with desirable properties is facilitated. We have studied two compounds which inhibit glutathione reductase as antimalarials: the clinically used antineoplastic agent carmustine (BCNU)[36,37] and its newly developed water-soluble and less toxic analogue, 1-(2-chloroethyl)-1-nitroso-3-(2-hydroxyethyl)urea(≙ HeCNU). Both drugs inhibited the growth of *Plasmodium falciparum* in culture. In addition, HeCNU was shown to have a curative effect on rodent malaria being

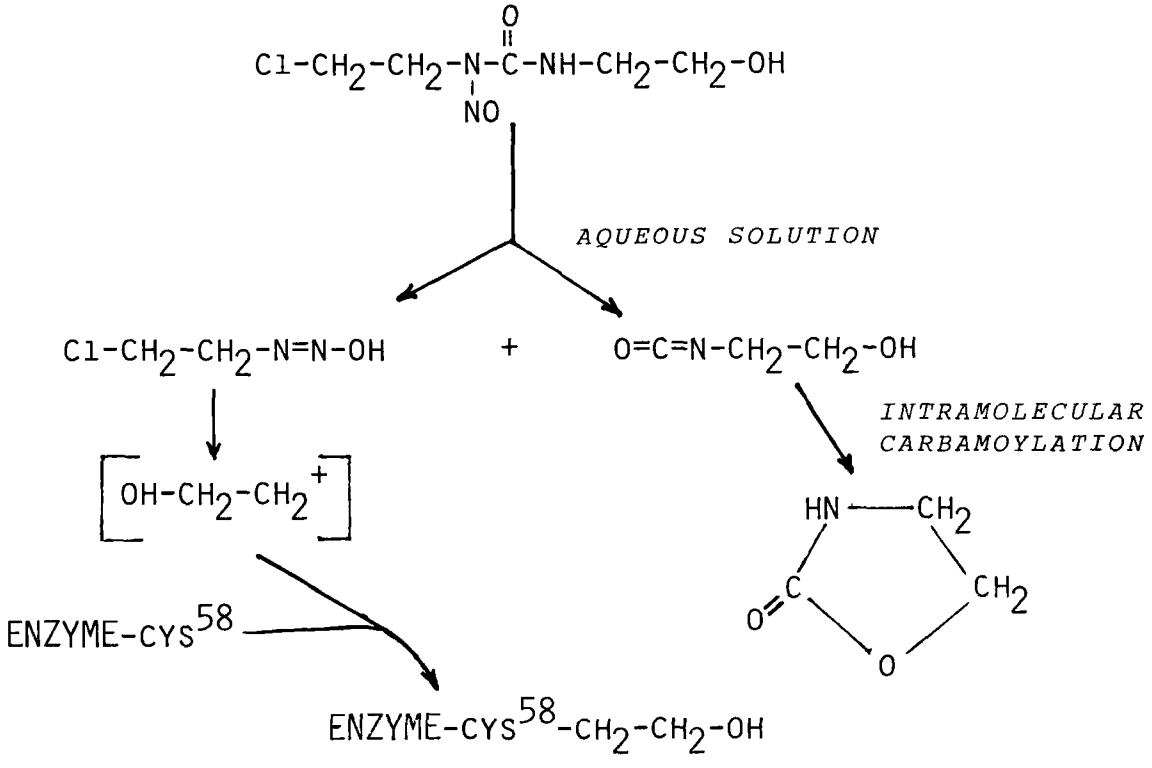

FIGURE 2 *Probable mechanism leading to inhibition of glutathione reductase by HeCNU.* HeCNU disintegrates in aqueous solutions to give hydroxyethyldiazohydroxide and hydroxyethylisocyanate. The latter cyclizes whereas the former yields alkylating species for the modification of Cys-58 (51). In the case of the BCNU-inactivated glutathione reductase a stable peptide derivative of the modified enzyme could not be isolated so far (ref. 37 and Krauth-Siegel, unpublished data). This suggests that BCNU modifies Cys-58 of glutathione reductase by carbamoylation via the chloroethylisocyanate, not by alkylation.

equally effective against chloroquine-resistant and chloroquine-sensitive strains of *P. vinckei*.[14]

Being carcinogenic, HeCNU is used as an antitumour drug but it might never be considered for treating human malaria. Nevertheless it is a valuable model drug whose mode of action may be of interest. As a first step we have studied the modification of purified human glutathione reductase by HeCNU *in vitro*. The experimental details are given under MATERIALS AND METHODS and in Fig. 2. Compounds like iodoacetamide and (the active derivatives of) BCNU and HeCNU[37,38] will inactivate glutathione reductase only after the active site disulfide Cys-58 – Cys-63 has been reduced. We used NADH rather than the physiologic substrate NADPH for this purpose.

When reduced GR was incubated with HeCNU, 0.94 residue of Cys-58 and 0.47 residue of Cys-2 were hydroxyethylated. As this led to 94% enzyme inhibition it is most likely that the modification of Cys-58 is responsible for the inactivation. The side reaction of Cys-2 – this residue is located on a flexible extension of the enzyme[17,33] – has probably no effect on the enzyme activity.

It is remarkable that in spite of the long incubation time of 46 h and the relatively high concentrations of HeCNU the other Cys residues of glutathione reductase were not hydroxyethylated. As inactivation of the enzyme by administration of HeCNU occurs more readily in intact red blood cells (König & Schirmer, unpublished results), our *in vitro* conditions may not simulate the *in vivo* situation appropriately. The working hypothesis, however, is as follows: In patients treated with HeCNU, Cys-58 of glutathione reductase is the major drug target. This residue is modified via an alkylating mechanism which involves the conversion of the chloroethyl moiety of HeCNU into a 2-hydroxyethyl group (Fig. 2). Approximately 50 ml erythrocytes from HeCNU-treated patients will be required for analyzing the modification of glutathione reductase which occurs *in vivo*.

OUTLOOK

Oxidative Stress and Malaria

The concept of oxidative stress may provide new approaches to the prevention and therapy of malaria. Inhibitors of antioxidant enzymes in combination with redox cyclers of the isouramil type are recommended by analyzing the biochemistry of favism and by other *experimenta naturae*. It should be noted that these protecting natural experiments refer to host proteins but it must be kept in mind that the erythrocyte and the parasite contain two different sets of antioxidant enzymes and that it might be possible to influence these enzymes differentially. This is certainly true for glutathione peroxidase[25] and might also be true for glutathione reductase. Thus drug design by receptor fit (DDRF) should aim at inhibitors of the parasite's glutathione reductase[39] which do not affect the host's GR and at inhibitors with reciprocal specificity.

Reactive oxygen species are among the effector molecules after active immunization: There is strong evidence that activated macrophages contribute, through ROS and other secretory products, to immunity against rodent malaria, and that vaccination can enhance this by focussing the cytotoxic attack where it is most needed.[3] This indicates that immunization and chemotherapy can overlap and complement each other.

In malaria tropica, parasitized red blood cells stick to postcapillary venules of the brain and elsewhere, thus avoiding the purgatory of the spleen and other macrophage-rich organs. For this condition compounds which activate the conversion of endothelial xanthine dehydrogenase to xanthine oxidase might be of special interest.[40]

Diseases Caused by Trypanosomes and Leishmanias

Trypanosomes and leishmanias are unicellular organisms which belong to the order Kinetoplastida. Many of them cause diseases of man and domestic animals, such as Chagas' disease, a very serious sociomedical problem in South America (caused by *Trypanosoma cruzi*), nagana in cattle (*T. brucei, T. vivax*), an obstacle to human welfare in Africa, oriental sore (*Leishmania tropica*) and kala-azar (*L. donovani*).

Recent studies have given rise to the expectation that oxidative stress might be the means of choice for the treatment of these diseases. This hope is based on the following results:

1) Trypanosomes are more sensitive to H_2O_2 and its derivatives than mammalian cells are.[5–7] In the case of T. brucei[7] it is phosphoglyceromutase, a glycolytic enzyme, which is inhibited at μmolar H_2O_2 concentrations.

2) Compounds such as nifurtimox[5,41,42] and naphthoquinones[5,43] that undergo intracellular one-electron redox cycling, and metalloporphyrins that accelerate the homolytic cleavage of H_2O_2 to form $\cdot OH$ radicals are trypanolytic *in vitro*. Nifurtimox – in the case of *T. cruzi* – and haematoporphyrin[44] – in the case of *T. brucei* – are also curative in trypanosomal diseases.

3) Protective enzymes such as catalase and glutathione peroxidase which occur in mammalian cells are absent in trypanosomatids.[5–7]

4) The antioxidant enzyme defense system of Kinetoplastida is based on trypanothione[45] and other glutathionyl spermidines,[46] not on glutathione as it is the case in their mammalian hosts. Thus there is a pathway, apparently unique to Kinetoplastida which may offer a new approach to rational drug design. A key enzyme of this pathway, trypanothione reductase, has been isolated form *Crithidia fasciculata*[47] and, more recently, from *Trypanosoma cruzi*.[48] H_2O_2 detoxification – as studied in *T. brucei* – involves NADPH and trypanothione[7] but details of the mechanism are unknown. Trypanothione peroxidase which was expected to be present could be identified so far neither in *T. brucei*[7] nor in *T. cruzi* (Schirmer, unpublished).

5) Considering glutathione and spermidine as the two components of trypanothione, it is interesting that inhibition of glutathione synthesis by buthionine sulphoximine,[49] or alternatively, inhibition of spermidine synthesis by administration of α-difluoromethylornithine (DMFO) are curative measures for early *T. brucei* infections in mice.[50] It is tempting to speculate that it is the continuous synthesis of antioxidants such as trypanothione and other glutathionyl spermidine compounds which makes polyamines essential for the multiplication and survival of trypanosomes in their vertebrate hosts.

The observation that in *T. cruzi* and *Leishmania* infections the organisms can multiply in macrophages does not speak against their susceptibility to oxidative stress. Indeed, the cytosol of macrophages is probably the space with the best equipment of antioxidant systems.[23,24]

Drug Design

We are interested in the development of oxidative stressors acting by more than one mechanism. These include compounds of the nifurtimox type which act both as redox cyclers and as inhibitors of the antioxidant enzymes glutathione reductase and/or trypanothione reductase.[38,48] Another type is represented by 2,4,6-trinitrobenzenesulfonate[52] and by paraquat.[53] In the presence of these substances GR and probably TR act as effective NADPH oxidases producing $O_2^{\cdot-}$ and H_2O_2.[52–54] This means that trinitrobenzenesulfonate and paraquat convert an antioxidant to a prooxidant enzyme. For compounds of this type we suggest the term turncoat inhibitors.

A combination of X-ray diffraction analysis and sequence analysis is expected to yield the 3-dimensional structure of trypanothione reductase in atomic detail.[47–48] Structural comparisons with human glutathione reductase, especially in the binding region of the disulfide substrates (GSSG in the case of GR, TS_2 and other glutathionyl spermidine compounds in the case of TR) should assist the design of specific drugs against trypanosomes and leishmanias. Many inhibitors of human glutathione reductase including the hormone triiodothyronine and the drugs nifurtimox, BCNU and HeCNU, are known.[38] In view of the structural and functional similarity between GR and TR, these inhibitors could serve as parent compounds when designing inhibitors of trypanothione reductase from Kinetoplastida, and of glutathione reductase from other parasites.

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