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Research review paper

The trypanothione system and the opportunities it offers to create drugs for the neglected kinetoplast diseases

Leopold Flohé *

Institute of Chemistry, Otto-von-Guericke-Universität, Universitätsplatz 2, D-39106 Magdeburg, Germany

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A B S T R A C T

Parasitic trypanosomatids (*Kinetoplastida*) are the causative agents of devastating and hard-to-treat diseases such as African sleeping sickness, Chagas disease and various forms of Leishmaniasis. Altogether they affect >30 Million patients, account for half a million fatalities *p.a.* and cause substantial economical problems in the Third World due to human morbidity and life stock losses. The design of efficacious and safe drugs is expected from inhibition of metabolic pathways that are unique and essential to the parasite and absent in the host. In this respect, the trypanothione system first detected in the insect-pathogenic trypanosomatid *Crithidia fasciculata* qualified as an attractive drug target area. The existence of the system in pathogenic relatives was established by homology cloning and PCR. The vital importance of the system was verified in *Trypanosoma brucei* by *ds*RNA technology or knock-out in other trypanosomatids, respectively, and is explained by its pivotal role in the parasite's antioxidant defense and DNA synthesis. The key system component is the bis-glutathionyl derivative of spermidine, trypanothione. It is the proximal reductant of tryparedoxin which substitutes for thioredoxin-, glutaredoxin- and glutathione-dependent reactions. Heterologous expression, functional characterization and crystallization of recombinant system components finally enable structure-based rational inhibitor design.

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* Rudolf-Breitscheid-Straße 184A, D-14482 Potsdam, Germany. Tel.: +49 391 6718673, +49 331 748 0950; fax: +49 391 67 12223.
E-mail address: l.flohe@t-online.de.

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1. Introduction

The therapy of infectious diseases caused by protozoan parasites of the trypanosomatid family is a neglected area of research and drug development (Fairlamb et al., 2003).These parasites comprise *Trypanosoma brucei rhodesiense* and *T. brucei gambiense*, the causative agents of African sleeping sickness, *T. cruzi* causing the Chagas disease in Latin America, and a large variety of *Leishmania* species causing *inter alia* the oriental sore (*L. major*), Kala-Azar or visceral Leishmaniasis (*L. donovani; L. infantum*) and the mucocutaneous Leishmaniasis "espundia" (*L. braziliensis*). Altogether they affect about 30 Million of people and account for half a million of fatalities *p. a.* They also cause substantial economic problems in endemic areas by affecting life stock (*e. g. T. brucei brucei, T. congolense, T. evansi*). Treatment of these diseases is unsatisfactory in terms of safety and efficacy, which sharply contrasts with the therapeutic needs in terms of people at risk, number of affected patients, and associated fatalities. This discrepancy is primarily due to the prevalence of these diseases in tropical and subtropical countries of partially poor socio-economic standards. Associated difficulties in market penetration at reasonable profit margins have dampened the engagement of pharmaceutical companies. In consequence, efficacious new drugs have not been developed, and available ones still comprise old-fashioned toxic arsenicals (Melarsoprol) and antimony-containing compounds (Pentostam), have unfavorable kinetics like Suramin or are unspecific redox cyclers damaging both the host and the parasite (e.g. Nifurtimox). The armamentarium has been enriched by compounds such as difluoromethylornithine (DMFO) and Miltefosin that originally were developed for cancer therapy. Otherwise, the pipeline of drugs for neglected diseases such as Leishmaniasis and Trypanosomiasis remained virtually empty (TDR, 2010).

Over the last decades, however, considerable efforts have been made in sequencing the genomes of most of the clinically relevant *Trypanosoma* and *Leishmania* species (Jackson et al., 2010; El-Sayed et al., 2005, Kissinger, 2006). These data bases provide ideal opportunities to identify metabolic pathways that are unique to the parasites (Myler, 2008). Further, methods of inverse genetics such as gene knock-out and knock-down strategies have been adapted to investigate the biological relevance of these pathways. It thus has become possible to define molecular entities ("targets") that are of vital importance for the parasite but absent in their mammalian hosts or sufficiently different to be targeted selectively. Any chemical entity that selectively binds to, and thereby inhibits, the molecular target of the parasite can be considered a potential drug to treat the parasite-infected patient or life stock, respectively. Moreover, such potential drugs can not only be expected to be efficacious but also safe, since they should not interfere with the host metabolism.

Unfortunately, related post-genomic research aiming at the functional characterization of potential drug targets is lagging behind (Myler and Fasel, 2008). Nevertheless, there is no longer a shortage of potential drug targets. The challenge rather is to select the most promising ones for drug discovery and development to make the most economic use of the still scarce resources. In this article a particular metabolic pathway, the trypanothione system that is unique to trypanosomatids, will be reviewed in respect to the opportunities it offers for therapeutic intervention.

2. Unraveling the trypanothione-dependent hydroperoxide metabolism

Trypanothione [N^1,N^8-bis(glutathionyl)spermidine; $T(SH)_2$], is a redox metabolite that has never been found in higher animals. It was detected in *Leishmania* and *Trypanosoma* species by Alan Fairlamb et al. in 1985 as substrate of a trypanosomatid "glutathione reductase" that, strange enough, preferred to reduce the cyclic oxidized form of the new glutathione derivative (TS_2) instead of oxidized glutathione (GSSG) (Fairlamb et al., 1985). The enzyme, like the real GSH reductase of other species, was characterized as a flavoprotein containing redox-shuttling thiols and, because of its divergent specificity, was called trypanothione reductase (TryR) (Shames et al., 1986). Still in 1986 $T(SH)_2$ was shown to be synthesized by stepwise ATP-dependent conjugation of GSH with spermidine, N^1-glutathionylspermidine being the intermediate (Fairlamb et al., 1986). While the latter compound had previously been detected in bacteria (Tabor and Tabor, 1983), $T(SH)_2$ proved to only occur in *Kinetoplastida* (Fairlamb and Cerami, 1992) and some other protists (Ondarza et al., 1999, Ondarza et al., 2006). It appeared to substitute for many of the multiple functions of GSH and thioredoxin in higher organisms (Fairlamb and Cerami, 1992). Accordingly, $T(SH)_2$ was from its discovery considered to be a promising target of trypanocidal drugs and, in support of this vision, some of the existing trypanocidal drugs were shown to interfere with the biosynthesis of $T(SH)_2$, DFMO by inhibiting spermidine synthesis and the arsenicals by binding to $T(SH)_2$ itself and/or toTryR (Fairlamb and Cerami, 1992). A role of $T(SH)_2$ in substituting for GSH could soon be corroborated for detoxification of xenobiotics and heavy metals (Fairlamb and Cerami, 1992), its relevance to peroxide metabolism, however, remained enigmatic for more than a decade.

Despite the advance of efficient molecular biology tools, all attempts to identify in trypanosomatids a trypanothione peroxidase that might be homologous and functionally equivalent to the mammalian glutathione peroxidases (GPx) (Flohé and Brigelius-Flohé, 2006) failed. The frustrating outcome of the struggle for the trypanothione peroxidase is reflected in a publication of the nineties claiming that hydroperoxide reduction by $T(SH)_2$ must be a non-enzymatic process (Carnieri et al., 1993). The enigma was solved in 1997 by Everson Nogoceke showing that the "trypanothione peroxidase" activity is not achieved by a single enzymatic entity but by two enzymes working in concert: *i*) tryparedoxin (TXN), a thioredoxin-related protein with the atypical active site motif CPPC which is specifically reduced by $T(SH)_2$; and *ii*) a tryparedoxin peroxidase (TXNPx), which is reduced by TXN. TXNPx turned out not to be a GPx-type protein but belonged to a then emerging new thiol peroxidase family, the peroxiredoxins (Nogoceke et al., 1997). Nevertheless, TXNPx, like the glutathione peroxidases, proved to be a broad spectrum peroxidase acting not only on H_2O_2, but also on organic hydroperoxides including complex lipid hydroperoxides such as phosphatidylcholine hydroperoxide (Gommel et al., 1997), and meanwhile it has become known that TXNPxs, like other peroxiredoxins, also reduce peroxynitrite (Trujillo et al., 2007).

The discovery of the trypanothione-dependent peroxidase system was achieved by conventional protein isolation and enzymological procedures taking advantage of the model organism *Crithidia fasciculata* which is apathogenic for humans and, thus, could be obtained in substantial quantities from large-scale fermentation. In retrospect, the reasons for previous failures to discover this enzymatic system are obvious. TXN is a small protein of ca 18,000 Da, while TXNPx is a decamer of >200,000 Da that tends to dissociate with loss of activity upon dilution. The two proteins are therefore readily separated and none of them shows any significant trypanothione peroxidase activity by itself. The second problem, the inactivation of TXNPx by dilution, was overcome in Everson's preparation by always keeping the enzyme at high concentration, as had, by serendipity, been achieved due to mass production of the starting material. With the first partial sequences of the two proteins, which had still to be done by Edman degradation (Nogoceke et al., 1997), the full length sequences were soon established by means of PCR and DNA sequencing (Guerrero et al., 1999, Montemartini et al., 1998a, Montemartini et al., 1998b) and the existence of the homologous system could be demonstrated in real pathogens such as *T. cruzi* (Lopez et al., 2000), *T. brucei* (El-Sayed et al., 1995, Lüdemann et al., 1998), *L. infantum* (Castro et al., 2004), *L. major* (Levick et al., 1998) and *L. donovani* (Flohé et al., 2002) by homology cloning.

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