The Thioredoxin System—From Science to Clinic

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Abstract: The thioredoxin system—formed by thioredoxin reductase and its characteristic substrate thioredoxin—is an important constituent of the intracellular redox milieu. Interactions with many different metabolic pathways such as DNA-synthesis, selenium metabolism, and the antioxidative network as well as significant species differences render this system an attractive target for chemotherapeutic approaches in many fields of medicine—ranging from infectious diseases to cancer therapy. In this review we will present and evaluate the preclinical and clinical results available today. Current trends in drug development are emphasized. Med Res Rev, 24, No. 1, 40–89, 2004

Key words: thioredoxin; thioredoxin reductase; selenium; cancer; inhibitor

1. INTRODUCTION

The cellular redox-milieu—with its metabolic, antioxidative, and regulatory aspects—is largely maintained and regulated by two enzyme-based systems: the glutathione and the thioredoxin system.[1–4] In each system, an NADPH-dependent flavoenzyme—namely glutathione reductase (GR; EC 1.8.1.7[a]) and thioredoxin reductase (TrxR; EC 1.8.1.9[b])—is involved. Both enzymes belong to a family of homodimeric pyridine nucleotide-disulfide oxidoreductases which further includes enzymes like lipoamide dehydrogenase, trypanothione reductase, and mercuric ion reductase.[5]

The classical thioredoxin system is formed by thioredoxin reductase and its characteristic substrate the redox active protein thioredoxin (Trx), whose reduction at the expense of NADPH is the naming reaction. Reduced thioredoxin in turn is reoxidized whilst providing reducing equivalents to target molecules (Fig. 1).

[a]Glutathione reductase was formerly designated 1.6.4.2; see: Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC IUBMB) (http://www.chem.qmul.ac.uk/iubmb/enzyme/EC1/cont1bb.html).
[b]Thioredoxin reductase was formerly designated 1.6.4.5.

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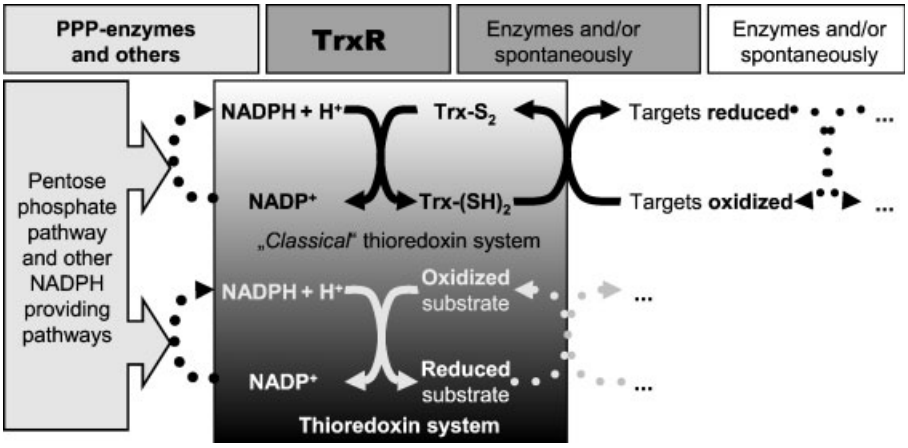

Figure 1. The thioredoxin system. The term classical referred to the functional unit of TrxR, Trx and NADPH. As a result of the increasing number of substrates that can be directly reduced by (mainly large) TrxRs, the meaning of this term has widened too. Even though being the major source of NADPH in the cytosol of most tissues, other sources than the pentose phosphate pathway (e.g., $NADP^+$-specific isocitrate dehydrogenase) may provide reducing equivalents, too—particularly in other subcellular compartments such as the mitochondria.[342]

Even though discovered in the nineteen sixties,[6,7] more than 50% of almost 2,700 thioredoxin-related entries found in the Medline database[c] today were published within the last 5 years. As most of the earlier studies were focused on the bacterial systems, where the cellular functions of these two proteins appear to be rather limited, the multitude of functions in mammalian cells had been largely ignored for many years. In 1996, thioredoxin research got a further boost when Stadtman's group discovered that mammalian TrxRs are selenoenzymes.[8,9] The number of published observations has dramatically increased ever since, but many findings still lack supporting experimental evidence regarding their physiological significance. However, available preclinical *in vitro* data and clinical data strongly support the notion that the thioredoxin system *is* of importance and that the development of drugs acting via the thioredoxin system is a promising route.

It is impossible to cover all aspects of the topic. In this article, we will briefly review the biochemical properties of the proteins involved with a special focus on their known intra- and extracellular functions and then discuss potential medical applications and aspects of drug development. Available associated clinical and preclinical data will be discussed and critically reviewed. Our list of references might serve as a basis for a more detailed view on certain aspects. In particular, we recommend the reviews given in Refs. 4, 10–17.

A problem occurring in all rapidly growing scientific fields, is the use of a non-homogeneous nomenclature as simultaneous discoveries lead to different names and terms for, in fact, identical things and processes. The thioredoxin field is unfortunately no exception to this rule. We have therefore used a more systematic nomenclature for different thioredoxins and thioredoxin reductases refered to in this article. It is based on the species name, the enzyme refered to (TrxR or Trx) and a numerical identifier. To facilitate species comparison, we used the human system as a standard. The human enzymes were ordered in the chronological order of their discovery (e.g., the human placental TrxR was discovered first, thus hTrxR-1) and the corresponding enzymes were assigned according to the human enzyme they resemble closest. Examples are shown in Table III, which may also serve as a reference to the terms used elsewhere.

[c]http://www.ncbi.nlm.nih.gov/entrez/

2. CLASSIFICATION OF THIOREDOXIN REDUCTASES

As the functions and biochemical features of the thioredoxin system in different species are quite diverse it seemed warranted to us to provide some basic guidelines first.

Thioredoxin reductase was first studied in *Escherichia coli.*[18–25] Soon it became clear, that the bacterial TrxR differs in many aspects from other members of the greater enzyme family. The most obvious difference is subunit size. Whereas the average subunit mass of glutathione reductases and lipoamide dehydrogenases (irrespective of the source) is around 55 kDa, the bacterial TrxRs exhibit an average molecular mass of 35 kDa per subunit.[5] In subsequent studies, it became evident that the catalytic cycle which even involves a domain rotation of 66° is distinct from all other members of the family.[17,26,27] Although certain common building units such as the NADPH and FAD binding sites are present in bacterial TrxR (Fig. 2), the sequence identities (Table I) and similarities are detectably low and it was not surprising that the 3-dimensional structure of *E. coli* TrxR differs significantly from related flavoenzymes.[28]

When the first mammalian TrxRs were purified, they turned out to be more typical members of the familiy. Subunit mass[29,30] and domain arrangement[31] as well as the mechanism of the reductive half reaction resemble that of glutathione reductase and lipoamide dehydrogenase.[32] Furthermore, unlike the small TrxRs whose substrate spectrum is very narrow, the large TrxRs exhibit a broad substrate spectrum that includes many different molecules apart from thioredoxin (e.g., Refs. 33–39)—a finding we will discuss later in more detail. Despite these striking differences, studies continue to be conducted or referred to using *E. coli* TrxR data for the interpretation of the mammalian system. It is clear that such data are highly susceptible to misinterpretations.

Initial classification attempts suggested the terms "*bacterial TrxR*" and "*mammalian TrxR*." However, TrxRs of the bacterial type are not present in the bacteria only but can also be found in plants,[40,41] fungi,[42,43] and some protozoa (as indicated by the presence of protein sequences. For example, *Pneumocystis carinii;* NCBI-accession no. AAN12366). Thus, the now more commonly used terms (Table II) are *small* TrxRs (subunit size approx. 35 kDa) and *large* TrxRs (subunit size approx. 55 kDa; CVNVGC as typical N-terminal active site sequence).

Until very recently it was believed that they are mutually exclusive, as it appeared that no organism harbored both classes of TrxR.[44] Novoselov et al., however, showed that there are indeed species—at least *Chlamydomonas reinhardtii*—which keep in possession both types of TrxRs.[45,46] Unlike earlier assumption, they suggest that it was an early eukaryote that possessed both classes of TrxR and that during the subsequent evolutionary steps either the small or the large TrxR was dropped in a phylogenetic branch.[45,46]

Whereas the class of small TrxRs is rather homogeneous, significant differences can be found among the large TrxRs. As a common structural feature, the large TrxRs have an additional C-terminal redox center that accepts reducing equivalents from the flavin-near cysteines and transfers them to the final substrate—yet the individual "styling" of this second redox center differs substantially (Fig. 3).

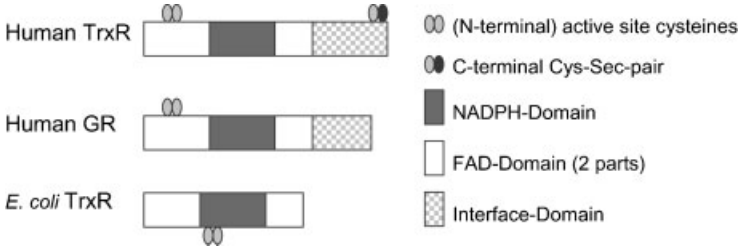

Figure 2. Domain organization of large and small TrxRs compared to glutathione reductase. Adapted from Ref. 39.

Table I. Sequence Identities Among Disulfide Reductases

	Human TrxR (large TrxR)	*P. falciparum* TrxR (large TrxR)	*E. coli* TrxR (small TrxR)	Human GR (*no* TrxR)
Human TrxR (hTrxR-1)	(100 %)	46 %	25 %	35 %
P. falciparum TrxR	46 %	(100 %)	23 %	29 %
E. coli TrxR	25 %	23 %	(100 %)	26 %
Human GR	35 %	29 %	26 %	(100 %)

When interpreting these data it must be taken into account, that certain functional building blocks (NADPH-binding site, FAD-binding site) show a high degree of sequence identity. The data show the closer relationship between GRs and large TrxRs in comparison to small TrxRs. Figures were calculated using LFASTA (using http://www.infobiogen.fr/services/analyseq/cgi-bin/lfastap .in.pl) based on Ref. 335.

A first surprise was the discovery that mammalian TrxRs are selenoenzymes.[8,47,48] Selenium is specifically integrated via selenocysteine (Sec) as the penultimate amino acid. It is part of the catalytically essential redox active sequence GCUG (where U represents Sec; see Figs. 2 and 3).[9] This finding explained the previously unsuccessful attempts to express the active enzyme recombinantly in full length[d].[31]

Since disulfide bonds between sequentially adjacent cysteines are normally strained (see Refs. 50, 51 and references therein), it is assumed that the selenocysteine—due to its approx. 15% longer bond[52]—is much more suitable. This view is supported by mutants in which Sec was replaced by Cys: These enzymes exhibit a dramatic loss in activity when compared to the wild-type enzyme (reported were approx. 1–11% residual activity. See Refs. 53, 54, 351). The large TrxR of *Plasmodium falciparum*—the causative agent of tropical malaria[55]—does not contain selenium although it shows, depending on the substrate, 25–93% of the specific activity of the human enzyme.[56–59] In this enzyme, the problem has been solved differently: instead of the Cys–Sec-motif an insertion of four amino acids between the two C-terminal redox active cysteines is used (Cys–GGGK–Cys). This allows to form a tension-free intramolecular disulfide bridge at the C-terminal active site without the need for selenium. The increased rotational flexibility caused by the ''glycine-spacer'' does, however, reduce catalytic efficacy, when compared to the human enzyme. These information in mind, the characterization of *Drosophila melanogaster* TrxR offered a surprise: this large, selenium-free TrxR possesses a Cys–Cys-motif at its C-terminal tail that—in contradiction to the initial theory—shows (depending on the substrate) approx. 50% activity of native human cytosolic TrxR-1.[60] The major differences between the two enzymes are the neighboring amino acids (Fig. 3): the flanking neutral glycines of the (known) mammalian TrxRs are replaced by polar serines—a peculiarity that is not limited to *Drosophila* but is apparently a common feature among the *diptera*[e] (Fig. 3; Ref. 352). Mutational studies show that it is the hydroxyl-groups of the serines which are responsible for the activity enhancement.[351]

[d]In Ref. 31, the authors also stated that the truncated protein lacking selenocysteine was unable to bind FAD. This, however, was not reproducible (Arnér and Holmgren, personal communication and own observations). In fact, the stable FAD-binding of the truncated enzyme species imposes a problem for the spectral analysis of the recently invented technology to express these selenoenzymes in heterologous systems.[49]

[e]Diptera: Insect order containing flies (e.g., gnats, mosquitoes, true flies). They are characterized by a single pair of membranous wings, a pair of halters (instead of the hind wings), sucking mouthparts, and a complete metamorphosis.

Table II. Features of Small and Large TrxRs

	Small TrxR	Large TrxR
Reduces (homologous) thioredoxin	**Yes**	
Reduces glutathione disulfide	**No**	
FAD-content per subunit[5,210,337]	1	
M_R per subunit[5,56,60,337]	approx. **35 kDa**	approx. **55 kDa**
DTNB reduction[34,338]	**only via thioredoxin**	**directly**
Substrate spectrum[34]	very narrow	very broad
Thiolate-FAD-charge transfer complex[32,339]	no	yes, very stable
Location of the cysteines relative to the flavin[32]	*Re*-side of FAD	*Si*-side of FAD
Conformational change during catalysis[17,26]	strong (66° domain rotation in *E. coli* TrxR)	assumed to be minimal
Reductive half reaction[32]	large differences to GR	like GR

Dark grey shaded lines indicate defining properties of all TrxRs. The features used to distinguish large and small TrxRs are shown in light grey. There are further differences between large and small TrxRs which are, however, not often used to differentiate between the two groups. Only one small TrxR known, namely that of the parasite *Giardia duodenalis*, is reported to be capable of reducing DTNB directly and therefore doesn't fit completely into this classification scheme.[336]

So far there is no generally accepted subclassification of large TrxRs, thus, we suggest to use the essential C-terminal sequence for clarification if needed (e.g., DmTrxR-SCCS in the case of *Drosophila melanogaster*, hTrxR-GCUG for the human or PfTrxR-GCG_3KCG for the plasmodial enzyme).

3. CLASSIFICATIONS OF THIOREDOXINS

A thioredoxin is defined as a protein of approx. 12 kDa that contains the active site sequence (W)**CGPC**(K)[f], also referred to as the "thioredoxin-motif." These active site cysteines form a disulfide which is reduced by the homologous TrxR (Figs. 1, 4, and 5). The reduced protein is classically capable of transferring the reducing equivalents to ribonucleotide reductase.[6,61] However, the functions of reduced thioredoxins are by far not limited to this reaction (Fig. 1), and in fact, different thioredoxins within one organism can serve different functions.[62,63]

The typical thioredoxin structure (Fig. 4A) consist out of five central β-strands, forming a twisted plane of β-sheets, surrounded by four α-helices.[64] The high degree of secondary structures may explain the high stability[65] of the protein towards, e.g., heat, even though exceptions do exit.[66–68] One

[f]Tryptophane (W) and lysine (K) are not generally accepted as being required for the thioredoxin motif. Occasionally even (G)lycine and (P)roline are questioned. However, we feel that—due to the already very broadly used term "thioredoxin, the core sequence CGPC should be considered a conditio sine qua non.

Enzyme	Species	NCBI access	C-terminal sequence	
hTrxR-1 (c)	Human	s66677	LSVTKRSGASILQA-**GC----UG**	Seleno-cysteine containing TrxRs
hTrxR-2 (mt)	Human	aad51324	LRISKRSGLDPTVT-**GC----UG**	
SsTrxR-1 (c)	Pig (*Sus scrofa*)	af277894	LSVTKRSGASILQA-**GC----UG**	
bTrxR-1 (c)	Bovine	o62768	LSVTKRSGGNILQT-**GC----UG**	
mTrxR-1 (c)	Mouse	np_056577	LSVTKRSGGDILQS-**GC----UG**	
mTrxR-2 (mt)	Mouse	nm_013711	LHISKRSGLEPTVT-**GC----UG**	
rTrxR-1 (c)	Rat	af220760	LSVTKRSGGDILQS-**GC----UG**	
rTrxR-2 (mt)	Rat	nm_022584	LHISKRSGLDPTVT-**GC----UG**	
CeTrxR-1 (c)	*Caenorhabditis elegans*	aad41826	TLEKKEGDEELQAS-**GC----UG**	
CrTrxR-2 (c)	*Chlamydomonas reinhartii*	aan32903	LEVTKRSGKSALKK-**GC----UG**	
DmTrxR (c/mt)	*Drosophila melanogaster*	aag25640	LAITKRSGLDPTPA-**SC----CS**	*Non* Seleno-cysteine containing TrxRs
MdTrxR (c)	*Musca domestica*	aac69637	LAITKRSGLDPTPA-**SC----CS**	
AgTrxR (c)	*Anopheles gambiae*	cad30858	LAITKRSGLDPTPA-**TC----CS**	
CeTrxR-2 (mt)	*Caenorhabditis elegans*	s15798	LHITKRSGQDPRTQ-**GC----CG**	
PfTrxR (c)	*Plasmodium falciparum*	caa60574	LFVTISSGLSYAAKG**GCGGGKCG**	
hTGR (c)	Human	aad39929	LEITKSSGLDITQK-**GC----UG**	Related enzymes
hGR	Human	rdhuu	LR	
SaHgR	*Staphylococcus aureus*	aaa98245	LKLAALTFDKDVSKL**SC----CAG**	

Figure 3. C-terminal sections of different large TrxRs as well as some closely related enzymes. Human glutathione reductase (hGR) is shown to indicate that the typical C-terminal tail of large TrxRs may be regarded as an extention to GR. Thioredoxin glutathione reductase (TGR)—initially attributed to the "pure" TrxRs—is an enzymatic chimera as it has TrxR and GR activity within one enzyme protein. Mercuric ion reductase (HgR) exhibits a C-terminal redox active site similar to that of large TrxRs, yet, the substrate spectra are very different: HgR's subtrate, Hg^{2+}-ions, inactivate TrxRs. Inversely, thioredoxin is not reduced by HgR.

of the two catalytically redox active cysteine residues (Cys32 and Cys35 in human Trx-1) is burried (Cys35), whereas the other is more solvent exposed (Cys32; Fig. 4B).[g]

The characteristics of thioredoxins are more conserved than those of the TrxRs, but there are differences. Some mammalian thioredoxins contain additional cysteines which are implicated in thioredoxin-dimer formation and other regulatory processes (e.g., Cys73 in human Trx, Fig. 4).[69,70] However, there are thioredoxins in mammals—mitochondrial hTrx-2—that resemble closely bacterial thioredoxins and lack these additional cysteines.[71] The fact that mitochondrial hTrx-2 is more closely related to the bacterial Trx than to the cytosolic isoform hTrx-1 is also of interest in the light of the endosymbiont hypothesis.

Human thioredoxin was identified as the active principle in very diverse functions and given different names since it was initially unknown that the new molecules are identical with thioredoxin.[72–74] Designations, such as "adult T-cell leukemia derived factor (= ADF),"[75] "early pregnancy factor (= EPF),"[74] or "interleukin-2 (IL-2) receptor-inducing factor"[76] should no longer be used for thioredoxin, yet they still remind us of some of the functions thioredoxin does exhibit in biological systems.

Interestingly, Trx-1[77] as well as Trx-2 gene[78] disruption experiments in mice resulted in embryonic lethality in homozygous animals, which indicates the importance of the thioredoxin system.

Apart from "normal" thioredoxins, truncated forms apparently play a significant biological role as well.[79] We will discuss this issue in a later section.

[g]As we believe that a more perceptible model may be beneficial for the understanding we recommend to envisage the thioredoxin structure as hamburger (Fig. 4B): one may regard the 5 β-sheets as the patty of ground beef covered by the roll formed by the 4 α-helices. The catalytically redox active residues—Cys32 and Cys35 in hTrx-1—would correspond to the tomato slice—partially visible, partially buried.

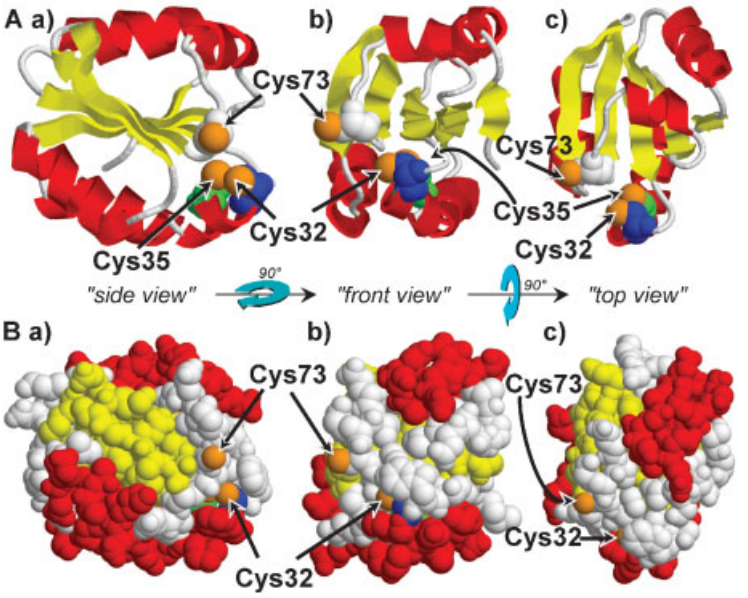

Figure 4. Structure of oxidized hTrx-1. **A**: Ribbon model (**B**) Space filling model. α-Helical structures are shown in red, β-sheets in yellow. The active site cysteines 32 (blue) and 35 (green) as well as the putative regulatory cysteine 73 (light gray) are indicated. The respective sulfur atoms are highlighted in dark orange. The protein is shown from three different perspectives: first (**a**) a view "*from the side*," (**b**) rotated 90° clockwise ("*front view*") compared to (a), and (**c**) an additional rotation by 90° downward ("*top view*"). The figure was created using RasMol V 2.7.2.1 (written by R. Sayle and H. Bernstein) and the PDB-dataset 1AUC.[343]

Moreover, there is a growing number of proteins that show sequence and structural similarity with thioredoxins (one of the most common protein domain folds)—in fact some even contain a redox active thioredoxin domain as part of the structure such as the recently discovered sperm cell specific proteins hSptrx-1.[80,81] In most cases, however, the redox active site is different, e.g., CG*H*C in protein disulfide isomerase (PDI), and calcium binding proteins 1 and 2 (CaBP1, CaBP2).[35,82] Many of these proteins can also be reduced by large TrxRs, yet they lack other defining features of a Trx—especially

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hTrx-1    ---MVKQIESKTAFQEALDAAGDKLVVVDFSATWCGPCKMIKPFFHSLSEKYS-NVIFLE  56
DmTrx-2   ---MVYQVKDKADLDGQLTKASGKLVVLDFFATWCGPCKMISPKLVELSTQFADNVVVLK  57
DmTrx-1   ----MASVRTMNDYHKRIEAADDKLIVLDFYATWCGPCKEMESTVKSLARKYSSKAVVLK  56
EcTrx-1   MSDKIIHLTDDS-FDTDVLKA-DGAILVDFWAEWCGPCKMIAPILDEIADEYQGKLTVAK  58
hTrx-2    --LTTFNIQDGPDFQDRVVNS-ETPVVVDFHAQWCGPCKILGPRLEKMVAKQHGKVVMAK  56
                :     .  :  :    :::** * ****** : . . .:  :   :  . :

hTrx-1    VDVDDCQDVASECEVKCMPTFQFFKKGQKVGEFSGANKE-KLEATINELV--        105
DmTrx-2   VDVDECEDIAMEYNISSMPTFVFLKNGVKVEEFAGANAK-RLEDVIKANI--        106
DmTrx-1   IDVDKFEELTERYKVRSMPTFVFLRQNRRLASFAGADEH-KLTNMMAKLVKA        107
EcTrx-1   LNIDQNPGTAPKYGIRGIPTLLLFKNGEVAATKVGALSKGQLKEFLDANLA-        109
hTrx-2    VDIDDHTDLAIEYEVSAVPTVLAMKNGDVVDKFVGIKDEDQLEAFLKKLIG-        107
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Figure 5. Alignment of different thioredoxins (using Clustal W version 1.82). The thioredoxin-motif (W)CGPC(K), typical for all thioredoxins is shown in bold. Additional cysteine residues have also been highlighted. NCBI-Accession numbers: hTrx-1:JH0568; mitochondrial hTrx-2:AAN05576.1[280]; DmTrx-1: P47938; DmTrx-2: AAF52794.1[63]; EcTrx-1: P00274. In the case of hTrx-2, the assumed mitochondrial importing sequence (residues 1–58) has been omitted.[280]

they do not provide reducing equivalents for ribonucleotide reductase. The casual use of the terms ''thioredoxin,'' ''thioredoxin domain,'' ''thioredoxin-motif,'' and particularly ''thioredoxin-like motif'' for proteins that lack the classical core CGPC sequence[83–85] and occasionally even Trx-typical redox-activity[83,85] is problematic.

Thus, only if a physiological *in vivo* interactions between the classical constituents of the thioredoxin system and those proteins are possible, these designations should be used and interactions taken into account when interpreting related data.

4. BIOLOGICAL ROLES OF THE THIOREDOXIN SYSTEM

The functions and actions of thioredoxin reductase and thioredoxin are impressive and it is virtually impossible to cover all aspects in a single paper. We would thus like to recommend also other reviews to the reader (e.g., Refs. 4, 10–13, 39, 86, 87).

A. Antioxidative Network

Cells must maintain a reducing intracellular milieu. The evolutionary emergence of molecular oxygen (O_2) imposed a serious threat to this essential condition. Even though many ''modern'' cells take advantage of the presence of oxygen, e.g., for energy production via oxidative phosphorylation, some chemical features of dioxygen remain a severe peril to cell integrity: normal (triplet-)oxygen ($^3O_2$) by itself is a weak, yet due to its abundance, a significant, paramagnetic diradical with two unpaired electrons. In activated states or as a result of unwanted side reactions, more *r*eactive *o*xygens *s*pecies (= ROS) such as superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), ozone (O_3) as well as the hydroxyl radical ($^{\bullet}OH$) emerge which can damage cellular constituents by oxidation.[88] Especially unsaturated carbon bonds in membrane lipids and cellular thiols are prone to such events generally leading to loss of function. Cells have developed enzymatic and non-enzymatic systems to reduce the concentration of these and other ROS.[89] Superoxide for instance is dealt with by superoxide dismutases that convert superoxide to hydrogen peroxide, which in turn is detoxified mainly by catalase[90] but also by glutathione and thioredoxin dependent peroxidases.[91,92] Even though these systems can handle huge amounts of reactive oxygen species, they are not perfect. Some reactive molecules will escape and damage potential targets which are chosen at random—any susceptible structure will do. Like in real life, cells recruit ''bodyguards'' and ''rescue specialists'' in case the ''bodyguards'' fail to protect jeopardized ''VIP-structures'' from oxidative stress: the major one of them is reduced glutathione (GSH) which is present in millimolar concentrations in most cells.[1] Due to its high concentration, it is much more likely that reactive molecules will react with the thiols of GSH than with the less concentrated thiols that have to be protected.[1] Thus GSH is sacrificed on their behalf. If, however, protein thiols should have been damaged, GSH is capable in many cases to repair this injury as well. To maintain continuous functionality, oxidized glutathione (glutathione disulfide, GSSG) is enzymatically reduced to GSH, mainly by the flavoenzyme glutathione reductase.[1] Apart from GSH, many other (however, normally less concentrated) low molecular weight compounds (e.g., ascorbic acid, vitamin E, ubiquinol, uric acid) provide similar protection. Often ignored as a major constituent of the cellular redox buffer are non-essential protein cysteines, which—when taken together—also provide thiols in the millimolar range.[1] When oxidized to sulfenates or disulfides they are mainly reactivated by GSH often in collaboration with glutaredoxin or by the other key player in the antioxidant game: reduced thioredoxin.

Oxidized glutathione is reduced rapidly in most cells by glutathione reductase which keeps up a molar ratio of around 1:600 for c(GSSG): 2 × c(GSH). Even though discovered quite early, the reduction of GSSG by reduced thioredoxin was considered to be negligible.[38,93,94] Thus it was even

more surprising that *Drosophila melanogaster*, one of the model organisms for *in vivo* studies, employs glutathione but lacks a typical glutathione reductase. It turned out that it was the thioredoxin system—previously considered to be negligible[38,93,94]—that kept glutathione in its reduced state in this insect.[58,60] Knock-out mutants of TrxR in *Drosophila* were found to be lethal.[95,96] First assumed to be a peculiarity of this particular organism it now appears that the lack of a genuine GR is a common feature among the *diptera* if not among insects (Refs. 60, 352). This is interesting since *Drosophila* and related organisms are readily exposed to oxidative stress: first of all, they need an efficient oxidative metabolism for providing sufficient energy to fly (which inevitably results in more side-reaction ROS[1]) and furthermore they expose their translucent wings to sunlight which adds ROS due to photo-oxidation. Apart from *diptera*, an increasing number of other organisms are now reported to lack a genuine GR—one of them is the human pathogen *Helicobacter pylori*.[97] To avoid misinterpretations, however, it must be added, that there are also organisms such as trypanosomes that apparently lack a genuine TrxR—even though thioredoxin is present.[98]

Apart from the Trx-driven GSSG reduction, other alternative pathways for the glutathione reductase independent reduction of oxidized glutathione have been suggested: for instance via the related glutaredoxin[h] and (predominantly enzyme-bound) dihydrolipoamide[99] or via TrxR and methylselenol.[100] However, a physiological *in vivo* role of these pathways is not yet established. If of relevance, the glutaredoxin-dihydrolipoamide pathway is presumably limited to the mitochondria, as the concentration of dihydrolipoamide is highest in this compartment, which also contains glutaredoxin.[101]

Like glutaredoxin and glutathione, the thioredoxin system is capable of regenerating proteins inactivated by oxidative stress.[102] In fact, the data presented indicate that the thioredoxin system may contribute more than 50% to this function.[102]

Thus the thioredoxin and the glutathione system, formerly considered to be clearly separated, turn out to constitute a balanced redox network in which functionality can be shared between the constituents to some extent. This view was emphasized by Burk et al. who reported liver and kidney necrosis when rats were depleted of glutathione under conditions of selenium deficiency, whereas selenium-sufficient control animals showed no necrosis.[103] A recently discovered human selenoenzyme—initially assumed to be a typical thioredoxin reductase (''hTR2'')[104]—is thioredoxin glutathione reductase (TGR).[105] In mammals it is testis-specific[105] whereas its distribution in other organisms is broader.[106] TGR may be regarded as a molecular chimera since it combines genuine (large) thioredoxin- and glutathione reductase activity in a single polypeptide chain.

Mammalian TrxRs as well as thioredoxin can recycle dehydroascorbate to ascorbate.[107] The data indicate an important role for the thioredoxin system dehydroascorbate reduction at least in liver, even though the glutathione system seems to contribute more to this function.[108,109] In a subsequent study, a TrxR-dependent ascorbate cycle was postulated for erythrocytes.[110,111] However, as pointed out later, we consider the TrxR-content (but not the Trx-content!) in human erythrocytes to be negligible.

Another finding is the *in vitro* reduction of ubiquinone to ubiquinol by mammalian TrxR, which is dependent on the presence of the selenocysteine.[112] However, this reduction is extremely slow (approx. 0.011 ubiquinone molecules per hTrxR-1 subunits and second), allowing the otherwise kinetically unfavorable reduction of hTrxR-1 by NADH.[113] Even though the provided cell culture data indicates that this activity may play a significant role *in vivo*,[112] an unambiguously direct *in vivo* link is missing. From the data provided,[112] it can be calculated that under selenite substitution the cellular ubiquinol formation is up to almost fourfold faster than *in vitro*, suggesting the formation of an intermediate such as methylselenol or another low molecular weight selenium compounds which links TrxR activity and ubiquinone reduction *in vivo*. A more selective

[h]Glutaredoxin (Grx) is also known as thioltransferase.

inhibitor of TrxR than Zn^{2+} used in the cited study might provide further insight into the *in vivo* situation.

B. Redox Regulation

Cellular functions need to be tightly controlled. A well-known and important control system is interconversion by reversible phosphorylation and dephosphorylation of target structures.[i] Another important regulatory principle is redox regulation.[114] The fact that cysteines in the active site of enzymes and proteins are normally essential for activity renders them ideal candidates for reversible modifications that allow a tight regulation of its action. A well-known example is the bacterial thioredoxin which in its reduced state serves as a subunit of the viral T7-polymerase, whereas the thioredoxin disulfide (despite only subtle differences in the crystal structure) does not.[115,116]

The concept of redox regulation requires that—despite the generally reducing cytosolic environment—oxidized species can also exist. Studies by C. Gitler and coworkers showed that under normal conditions approx. 11% of the cellular thioredoxin is present in the oxidized state.[117] As oxidation is most often due to an increased flux of (mainly exogenous) ROS, it seems natural that redox regulation acts primarily as a response mechanism to this oxidative stress.[118,119]

Examples of such redox-regulated processes are the activation or deactivation of enzymes (see e.g., Ref. 120) and several transcription factors (see Ref. 121 for a review). Many of these factors have either been shown or are assumed to be redox-regulated by thioredoxin.[119,122–138]

Interestingly, hTrxR-1 expression itself appears to be linked to the cellular redox-milieu.[139] The core promoter exhibits the typical features of a housekeeping gene, yet due to the abundance of AU-rich elements, hTrxR-1-mRNA-levels can be post-transcriptionally regulated. This regulation process is apparently redox-sensitive.

NF-κB is a frequently studied example of thioredoxin redox regulation. NF-κB, discovered in 1986, is a ubiquitous family of inducible transcription factors. It is beyond the limits of this review to explain this complex and yet not fully understood system, but we feel that a few aspects need to be pointed out. For more detailed reviews on NF-κB, see Refs. 140–142. NF-κB, which is activated by a large variety of stimuli, mainly as the result of inflammatory cytokines, infections, carcinogens, ROS, cellular stresses, and apoptosis inducers, controls approx. 180 genes. The gene products are diverse, yet many have antiapoptotic effects or are otherwise ''beneficial'' for uncontrolled cell growth. Lack of NF-κB leads to immune defects. In humans five different subunits of the NF-κB complex are known today: NF-κB_1 (p50), NF-κB_2 (p52), RelA (p65), RelB, and c-Rel. The main form of ''NF-κB'' is made up by heterodimerization of RelA and NF-κB_1 (p50). Their translocation into the nucleus and thus their activity as a transcriptional factor is, however, inhibited by $I\kappa B_\alpha$. At least NF-κB_1 (p50) requires a specific cysteine residue (Cys62) in the reduced form for efficient DNA-binding and thus for activity. Cys62-reduction is performed by thioredoxin.[143,144] To make things more complicated, thioredoxin seems to act differently in the cytosol and in the nucleus, as it can also inhibit TNF-α-induced or IL-1-induced NF-κB activation by interacting with the signaling cascade required to remove the inhibiting factor $I\kappa B_\alpha$, preventing the nuclear translocation and DNA-binding of the active NF-κB complex.[136,142,145]

The activated form of the tumor suppressor p53 can lead to apoptosis.[146,147] The thioredoxin system has been implicated in the activation of p53 by enhancing its DNA-binding capability.[125,134,148–151] However, the p53–thioredoxin interaction data are yet not fully consistent. Interestingly, activated p53 repressed TrxR transcription and expression as reported recently by Gladyshev and coworkers.[152] Taking further into account that a functional mitochondrial thioredoxin

[i]The human genome encodes presumably more than 600 kinases and approx. 30% of all proteins are phosphorylated. M. Wiese, personal communication.

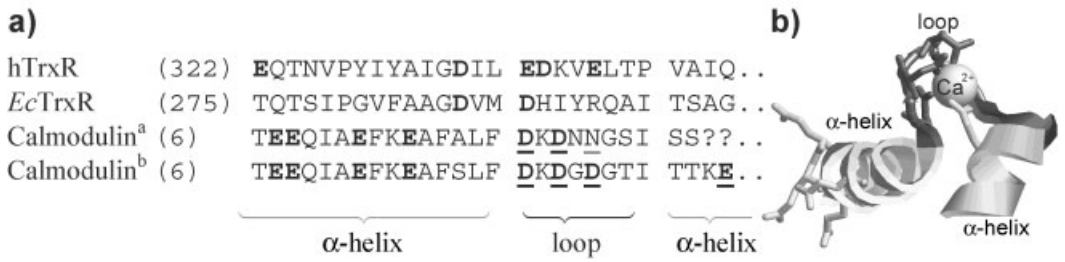


Figure 6. Alignment of TrxRs with calmodulins. In the original publication, *E. coli* TrxR and calmodulin[a] were aligned as shown.[158] No information is given on which calmodulin was used as reference. Added are the sequences of human calmodulin[b] (NCBI accession number: p02593) and the homologous part to the *E. coli* sequence in human TrxR (NCBI accession number: s66677) as calculated using ClustalW. Indicated in bold are amino acids with negatively charged side chains. Underlined are residues in calmodulin that do bind calcium. The designation to the secondary structures (α-helix and loop) refers to the calmodulins. The TrxR-sequences that correspond to the first α-helix in the calmodulins are in fact mainly β-strands. It becomes obvious that both large and small TrxRs have little in common with calmodulins and lack essential calcium binding residues present in calmodulins. Protein database code CLL.[344]

system prevents cells from undergoing apoptosis,[153–155] it is likely that the high Trx and TrxR contents in tumor cells indeed prevent apoptosis.

Ca^{2+}-ions exhibit a dose dependent inhibitory effect on hTrxR activity in the physiological cytosolic concentration range (Refs. 156, 157 and Gromer S., unpublished observation), whereas Mg^{2+} does not (Gromer S., unpublished observation). Supporting evidence for a physiological relevance of these Ca^{2+}-effects comes from Gitler's group[117] who reported a parallel increase in the concentrations of oxidized cellular thioredoxin and calcium.

In the initial reports it was stated that this inhibition by Ca^{2+} was not reversible by calcium chelators such as EGTA—a finding hardly compatible with a regulatory function. It was not reproducible in our hands.

An EF-hand (the name derives from parvalbumin, where calcium-binding takes place in a loop with many negative charges between two α-helices named E and F) has been reported to be present in *E. coli* and human TrxR by Schallreuter et al.[158] This is, however, not the case (Fig. 6): the sequence alignment did not only use the *E. coli* (small) TrxR as search model but also identified an ''EF-hand'' with only two negatively charged residues. Several negatively charged residues are, however, essential for binding Ca^{2+} in the EF-hand loop (Fig. 6). When looking at the crystal structure of reduced and oxidized *E. coli* TrxR as well as mammalian TrxR, no EF-hand can be found, nor any other related motif that could easily explain the observed phenomenon.

C. The Synthesis of Deoxyribonucleotides

All cells that multiply must prepare an identical copy of their DNA—a task that requires the presence of deoxyribonucleotides. The deoxyribonucleotides are formed from ribonucleotides by reducing the hydroxyl group at the C2′ of the ribose-moiety. This reaction is performed by ribonucleotide reductase and requires reducing equivalents which can normally be provided by either glutaredoxins (Grx) or thioredoxins:[34]

$$NDP + Grx-(SH)_2 \rightarrow dNDP + H_2O + Grx-S_2$$

or

$$NDP + Trx-(SH)_2 \rightarrow dNDP + H_2O + Trx - S_2$$

It is this reaction which led to the discovery of thioredoxin.

The question to what extent either of these systems contributes to the formation of desoxyribonucleotides *in vivo* is not yet fully resolved and it is possible that at least in some tissues other providers (e.g., via TGR in testis?) may contribute. Furthermore, differences between tissues and species may exist. From a more theoretical point of view, it may be argued that the *in vivo* TrxR activity (>1 U/ml) meets the demand for DNA building blocks, which is as high as 10 μmol/g cells in each cycle. In mouse fibroblast cultures, Spyrou and Holmgren were able to show that pharmacological GSH-depletion of the cells did not alter the rate of DNA-synthesis nor reduce the desoxyribonucleotide pool.[159] This may be regarded as indirect evidence for the importance of the thioredoxin system at least in fibroblasts.[159] The reliability of this interpretation is somewhat weakend by the reported low concentration of TrxR in rodent fibroblasts as determined in an enzyme-linked immunosorbent assay (ELISA) using anti-(*human*-TrxR)-IgG.[160] We assume, however, that this was most likely due to the low interspecies cross-reactivity of the anti-(*human*-TrxR)-IgG, as the concentrations for liver, known to contain large amounts of Trx and TrxR,[30,47,161] given in the cited report are low as well. To our knowledge, this question of the relative importance of either system for the deoxyribonucleotide synthesis (despite its importance) has never been addressed directly.

In rats, a study by Hansson and coworkers showed little correlation between the localization of thioredoxin and the M_1-subunit of ribonucleotide reductase.[162] There may be one—even though very speculative—explanation for this unequal distribution of Trx and ribonucleotide reductase: a direct reduction of ribonucleotide reductase by large thioredoxin reductases—a possibility that has so far not been taken into consideration.

D. Involvement in Hormone Action and Cytokine Function

The constituents of the thioredoxin system have been implicated in many aspects of hormone action and cytokine function.

Reduced thioredoxin was found to be secreted from cells to the extracellular compartment by an unusual pathway. It can act as an autocrine growth-factor synergizing with IL-1 and IL-2.[163] Its precise mode of action remains to be clarified as no receptor has been identified so far—even though protein kinase C appears to play a role in this process.[164] The data also indicate, that the redox properties of Trx are involved in autocrine stimulation.[165] However, a truncated form of Trx (Trx80/ECEF) is also secreted by many cells and acts as a mitogen for peripheral mononuclear blood cells, yet apparently lacks the redox properties typical for Trx.[79,166]

Intracellularly, thioredoxin acts as a reducing agent for several transcription factors, such as NF-κB and the glucocorticoid receptor. However, the functional effect of reduction differs from factor to factor: Trx-dependent reduction of NF-κB (p50 subunit) promotes enhanced *DNA* binding, whereas thioredoxin-mediated reduction of the glucocorticoid receptor enhances the receptors ability to bind the *hormone*.[167] Interestingly, the glucocorticoid receptor also requires (an additional) reduction to allow its binding to DNA which is, however, not due to Trx.[168]

There is furthermore evidence that the thioredoxin system is required for iodothyronine 5′-deiodination by deiodinase with reduced thioredoxin acting as an enzyme activator.[169]

E. Protein Biosynthesis

The thioredoxin system has been reported to maintain the high activity of the rat reticulocyte protein biosynthesis machinery. The glutathione system was not capable of replace it in this function.[170,171] The precise mechanism of this process has, however, not yet been studied in more detail.

Mammalian TrxRs require selenium for their synthesis. Increasing the selenium pool leads to an increase in TrxR activity until saturation is reached.[172–174] To what extend the selenium dependent synthesis of mammalian TrxR—if at all—therefore regulates the general level of protein synthesis, is not known.

5. LOCALIZATION, DISTRIBUTION, AND CONCENTRATION

Even though essential for the correct interpretation of *in vitro* data, well-validated information on local concentrations, tissue distribution, and subcellular localization of thioredoxin and thioredoxin reductase is still missing. The reports are often contradictory. One reason for this might be a recently reported heterogeneity within a TrxR population which may be of importance for subcellular localization and even function.[175,176] To complicate things even further a heterogeneous nomenclature is used for the different TrxRs especially in mammals. As pointed out before, we use a more systematic nomenclature for the different TrxRs and Trxs throughout this article as described in Table III (adapted from Ref. 39).

As a guideline Trx is an ubiquitous protein with concentrations between 1 and 20 μM (Table IV) even though concentrations of up to 100 μM[177] and tissue specific isoforms[80,178,179] have been published. TrxR concentrations are calculated to be around 1 μM in the cell.[3]

It is generally agreed that thioredoxin reductase activity is found in the cytosol and in mitochondria.[180–184] Thioredoxin occurs in all subcellular compartments, with considerable affinity to virtually all cellular membranes.[180,185,186] Thioredoxin reductase has also been reported in all subcellular compartments[187] and found to be membrane associated.[188] However, a definite answer as to whether TrxR must be considered a real membrane protein with remaining Trx-reducing function is still missing. This question is of significance since many effects found for extracellular functions of thioredoxins and other substrates are dependent on their redox state. As the extracellular (as well as the endoplasmatic reticulum's) milieu is oxidizing, the question must be raised if proposed functions such as vitamine K reduction,[189,190] plasma glutathione peroxidase reduction,[191] NK–lysin inactivation[192], etc. are of physiological importance if no reducing enzyme (and reducing equivalents) should be around (see Ref. 189 and references therein as an example for the problems that arise from this lack of knowledge in interpreting data). Schallreuter and Wood' study indicates that mammalian thioredoxin reductase is capable of reducing an obligatory extracellular quaternary ammonium compound in tissue biopsies.[188] Even though these findings have not been reproduced by others and the specificity of this assay has been questioned,[193,194] the results may represent the first evidence for transmembrane TrxR-activity *in vivo*. Recent immunohistochemical studies support the presence of a TrxR associated with the plasma membrane,[160] yet its precise localization and functional orientation remains to be established.

Information on tissue specific expression varies greatly, which may in part be due to the methods used but apparently significant species differences do exist as well. As judged from mRNA-levels in normal cells mitochondrial hTrx-2 and hTrxR-2-levels (Table III) correlate,[182] whereas hTrx-1 and hTrxR-1 levels do not.[195] One technical problem originates from using highly specific antibodies which may have led to false negative results for either Trx or TrxR if the investigator was unaware of the existence of isoforms within the same cell. Furthermore, some studies deal with mRNA-levels but do not take into account that they may not correlate directly with protein levels (see Fig. 2 in Ref. 104 for an example). This should be kept in mind when interpreting the available data. When browsing through the given information, it becomes clear that new studies on the protein level with all isoforms of Trx and TrxR are urgently needed.

A. Blood Cells and Blood Plasma

Thioredoxin has been found in erythrocytes in considerable concentrations (see Ref. 180 and Table IV). Thus it appears reasonable to expect the reductase as well. Cha and Kim reported the identification of a thioredoxin reductase in human erythrocytes.[196] Their first evidence was an immunoblot using polyclonal antibodies raised against *E. coli* and yeast thioredoxin reductases—both members of the *small* TrxR-family—to demonstrate the presence of a *large* thioredoxin reductase in extracts of human erythrocytes. We and others were, however, unable to

Table III. Nomenclature Used for TrxRs and Trxs in This Review

Used in this review	Equivalent in earlier publications	Chromosomal position in humans	NCBI accession number
hTrxR-1	TrxR1 [182] TrxR α [340] hTR1 [104]	12q23-q24.1 [195]	s66677
hTrxR-2	TrxR2 [182] TrxR β [340] hTR3 [104]	22q11.2 [182]	np_006431
hTGR	hTR2 [104] TGR [105] TrxR3 (unpublished; aad39929)	3q21.2	aad39929
hTrx-1	ADF [72] Thioredoxin-1 [11]	9q32 [341]	jh0568
hTrx-2	hTrx2 [153]	22q13.1	nm_012473.3
hSptrx-1	Sptrx-1 [80,178]	18p11.2-11.31 [178]	aak94950

The abbreviations used here are designed as follows:
Species (e.g., h = human, r = rat, m = Mouse, Dm = *Drosophila melanogaster*). TrxR or Trx.
Numeric identifier. In case of human enzymes, in historical order of discovery. The enzymes of other species should be designated according to the human isoenzyme they resemble most. This should facilitate species comparison. Examples.

Table IV. Concentrations of Thioredoxins in Various Tissue Homogenates (1-Week-Old Calf)

tissue	liver	kidney	thymus	spleen	lung	tongue	brain	heart	erythrocytes	platelets	plasma
[Trx] in μM	10	5	3.8	2.9	2.6	2.6	2.1	1.3	3.1	2.5	0.0042

As unfractioned tissues were used, local and subcellular differences in concentration are not taken into account. Thus, significant local deviations from these somewhat theoretical values must be borne in mind. Taken and calculated from Ref. 180.

detect a reactivity between anti-(*E. coli* TrxR)-antibodies and highly purified human TrxR and *vice versa*.

Neither fresh lysates from human erythrocytes nor 2′,5′-ADP-sepharose enriched samples showed reactivity in immunoblots using anti-(human-TrxR)-antibodies. Our anti-(human TrxR)-antibodies were carefully deprived of glutathione reductase cross-reacting immunoglobulins. This is of importance, as many preparations of TrxR are contaminated with small amounts of glutathione reductase. This glutathione reductase contaminations often result in the formation of significant amounts of anti-(glutathione reductase)-antibodies in the immunized animal. These leads to false positive reactions in immunoblot of samples such as red blood cells extracts which contain glutathione reductase in abundance. Furthermore, the DTNB-reduction activity Cha and Kim had found[j] in erythrocytes depended on the presence of thioredoxins—a feature of *small* TrxRs. We have analyzed (Gromer S, unpublished results) fresh lysates and 2′,5′-ADP-sepharose enriched extracts from human erythrocytes by cellogel electrophoreses and were unable to detect significant direct DTNB-reduction or a thioredoxin-reducing activity. Many subsequent studies have relied on Cha and Kim's paper when interpreting their data.[110,111,160] As others also reported a very weak TrxR-immunohistochemical reactivity of human erythrocytes,[160] we cannot fully rule out the existence of some TrxR-activity in erythrocytes, yet it can be concluded that the concentration of the enzyme is extremely low. It must furthermore be taken into account, that—given the concentrations in the cell—thioredoxin and glutathione can reduce each other (Ref. 197 and H. Merkle, personal communication). Thus, oxidized thioredoxin can be reduced by GSH which may functionally replace a TrxR in erythrocytes.

Rat megakaryocytes and platelets showed moderate to high immunoreactivity for Trx and TrxR, as did plasma cells whereas lymphocytes showed little if any reactivity.[161] However, in immunohistochemical TrxR-staining experiment of human blood cells lymphocytes showed moderate reactivity and platelets were negative. Monocytes gave strong signals, whereas granulocytes showed little to moderate staining.[160]

Thioredoxin is actively secreted in its reduced state by normal and neoplastic cells through an unusual secretory pathway.[198–200] However, blood plasma Trx concentrations are low (Table IV). Unless it is effectively re-reduced (e.g., by a membrane-associated enzyme) it is unlikely to plays a significant role as a reductant, e.g., plasma glutathione peroxidase[191] as it has been suggested. However, it apparently does serve as a cytokine—especially in its truncated form.[79,166]

Also, thioredoxin reductase has been reported to be secreted into the blood plasma.[160,201] Unlike thioredoxin, thioredoxin reductase is secreted via the classical Golgi-pathway.[160] The reported concentrations (approx. 160 pM) are, however, even lower than those of thioredoxin (approx. 4.2 nM). Even though it is tempting to speculate that a completely extracellular thioredoxin system could work, one must take into account that a *sufficient* supply of NADPH (or another effective reductant) is also required.

B. Heart

Rozell and Holmgren (unaware of the existence of rTrxR-2 and rTrx-2 at that time) found the rat heart muscle to be essentially negative in immunostaining for TrxR and Trx.[161] However, Spyrou et al. in fact cloned mitochondrial rTrx-2 from rat heart and also showed high mRNA levels.[185] In a recent clinical case-report, Shioji et al.[202] reported the results of thioredoxin immunostaining in cardiac biopsies of a patient suffering from myocarditis (using antibodies raised against a hTrx-1 fragment).

[j]It is difficult to calculate activities using the data of the report. After $(NH_4)_2SO_4$-precipitation, DEAE-cellulose, Sephacryl-200 and Blue-Gel the specific activity is given to be 81.31 ΔA_{412nm} per mg and min at 37°C. From this data we calculated that this correlates to 1 U/mg at 25°C, compared to 40 for the purified TrxR from human placenta. Even if we assume that the purified activity is only due to 25% of the total protein, this would only increase the specific activity to 4 U/mg.

Whereas the unaffected parts of the heart were almost negative, affected sites showed strong staining, which was due to both—inflammatory cells and myocytes. Similar results were obtained in the rat animal model of giant-cell myocarditis.[203]

TrxR-activity has been demonstrated for the murine heart [204,205] and mRNA levels in humans and mice suggest significant expression of hTrxR-1 and hTrxR-2.[104,182,206] The presence (or absence) of TrxR in the heart muscle may be of importance since for example the clinically commonly used anthracyclines (Fig. 18) such as adriamycin—known for their cardiotoxic effects—have been reported to act as rat TrxR-inhibitors.[207–209] The published inhibitory effects were only partially reproducible in our hands when using isolated cytosolic hTrxR-1.[210] It is interesting to note that selenium supplements ameliorate the cardiotoxic effects of adriamycin in rats.[211] After analyzing the presented figures, we believe, however, that one has to be cautious not to overinterprete the cardioprotective effects.

C. Liver and Gall Bladder

The liver parenchyma is probably the only tissue where all authors can agree on. Thioredoxin and thioredoxin reductase were demonstrated in large amounts in rat hepatocytes, with enrichment in the plasma membrane zone. In contrast, sinusoidal lining cells were found to be negative.[161] Western blot analysis showed the presence of both isoforms, rTrxR-1 and rTrxR-2,[205] and Northern blot analysis of the human organ resulted in similar results.[182] Mitochondrial mTrxR-2 is also expressed in mouse liver, although at much lower concentrations when compared to the predominant isoform mTrxR-1.[175] Ultrastructural investigations of rat liver showed a particularly dense association of Trx and TrxR with the granular endoplasmic reticulum and the cisternae of the Golgi complex but these proteins were present all over the cytosol and in the nuclear chromatin.[184] Bile ducts and gall bladder were studied only by Rozell et al. in 1985 who found a slight to moderate reactivity with Trx but not TrxR.[161]

D. Spleen

The immunohistochemically investigated rat spleen was essentially negative for Trx and TrxR in the medulla but subcapsular reticular cells and some plasma cells were intensely stained for both enzymes.[161]

E. Thymus

Interdigitating cells in the thymic medulla are immuno-positive for thioredoxin (hTrx-1).[212] Holmgren and Luthman report that thioredoxin concentrations in thymus ranked third after liver and kidney in bovine tissue.[180] Northern blot analysis of human thymic tissue indicated, however, a low transcriptional level for both TrxR-isoenzymes (hTrxR-1 and hTrxR-2).[182]

F. Thyroid and Parathyroid Gland

The rat follicular cells exhibit large variations in Trx and TrxR reactivity which reflects their metabolic activity—the most active cells are stained most intensely. Parafollicular cells and the parathyroid were also intensely stained.[161] The expression level of TrxR in thyrocytes is affected by the calcium-phosphoinositol signaling pathway, which presumably counteracts increased peroxide levels during hormone synthesis.[213] The presence of thioredoxin dependent peroxidases supports this view.[214]

G. Adrenal Gland

Rozell reports low to moderate staining for Trx in the rat adrenal cortex and strong reactivity in the medulla. TrxR-reactivity was moderate to strong in either part.[161]

H. Digestive Tract and Pancreas

The epithelium of the complete rat intestine as well as the pancreas show light to moderate reactivity for Trx and TrxR with a pronounced signal in the small intestine and APUD cells. In gastric parietal cells, the reactivity for thioredoxin was high. The exocrine Paneth's cell of the small intestine and pancreatic D-cells were most intense for TrxR. The *Peyer* patches showed intense staining caused by plasma and M-cells. Trx and TrxR have been immunohistochemically demonstrated in the periphery of the cytoplasm and in cytoplasmic granules of acinar and islet cells in mouse pancreas.[161] The staining for thioredoxin was more intense in the exocrine acinar cells than in the islet cells of animals fed *ad libitum*. Thioredoxin reductase was more intense in the endocrine (especially D-)cells. Fed animals showed low reactivity in the exocrine pancreas whereas starved rats exhibited an increased signal for both enzymes in the membranous area around vesicles.[215] Northern blot analyses of human tissue are contradictory. Whereas Miranda-Vizuete and coworkers report only a faint mRNA level of hTrxR-1 in the small intestine and an average level for the colon,[182] Gasdaska et al. report higher levels for hTrxR-1 in small intestine than in the colon.[195] The reported mRNA-levels for mitochondrial hTrxR-2 are comparable.[182,195]

I. Respiratory System

Nothern blot analysis revealed average mRNA levels for hTrxR-1 and hTrxR-2.[182] Immunohistochemical analysis of rat tissue showed high reactivity for both thioredoxin and its reductase in APUD-cells and to a lower degree in respiratory bronchiolar epithelial cells and great alveolar cells. Alveolar lining cells, however, showed no reactivity.[161] mRNA-levels determined in humans indicate a predominance of hTrxR-1 in this tissue.[182] Interestingly, both enzymes, TrxR and Trx, are upregulated upon oxygen breathing at birth.[216]

As shown by several studies, tumors of the lung and the mesothelium exhibit highly increased levels of both enzymes, too.[217,218]

J. Salivary Glands

Duct cells of rat salivary glands show increasing levels of either protein with increasing diameter. Mucous acinar gland cells showed only marginal staining and serous cells a low intensity staining.[161]

K. Skeletal and Smooth Muscle

Like in the case of heart muscle Rozell and Holmgren[161] found these rat tissues—with the exception of the genital tract—to be essentially negative. The positive result for the uterus was confirmed in the work of Lee et al.[205] In contrast to Rozell's data, Northern blot analysis of human skeletal muscle showed strong signals for both hTrxR-1 and hTrxR-2.[182]

L. Bone, Cartilage, and Tendons

For these tissues, only the rat immunohistochemical data are available,[161] which showed moderate staining for chondrocytes only, whereas genuine bone tissue, tendons, and periosteum were found to be negative.[161] Yet in human fetal osteoblasts a significant TrxR[219] and Trx[220] activity has been determined.

M. Skin

Stratum germinativum, hair follicles, nail beds, and sweat glands in rat skin showed a moderate to intense immunoreactivity for Trx and TrxR. Also Langerhans' cells and melanocytes exhibit a strong reactivity for either protein. Keratinizing cells, however, were only positive for thioredoxin.[161]

Schallreuter and Wood purified thioredoxin reductase from human melanoma metastases[188] and report varying specific activity in several skin disease.[221,222]

Selenium supplement in the media of several human skin cell lines (fibroblasts, keratinocytes, and melanocytes) leads to an increased synthesis of TrxR but also of phospholipid glutathione peroxidase which was found to be protective against UVB-radiation induced cell death.[223] It should, however, be pointed out that Clark et al. did not find a reduction in skin tumor rates after selenium supplements (yet incidences dropped markedly for other tumors such as prostate cancer).[224]

N. Kidney and Urinary Tract

Immunostaining by Rozell et al.[161] of the rat urinary tract showed a low to negative reactivity. Only the parietal cells of Bowman's capsule and tubular cells exhibit a somewhat increased reactivity.[161] This is in contrast to more recent publications that reported mRNA levels in humans, where relatively high mRNA levels for hTrxR-1 and hTrxR-2 are listed.[182] A current immunohistochemical study of normal rat kidneys reported high reactivity for Trx, rTrxR-1, and rTrxR-2 in proximal and distal tubular epithelial, papillary collecting duct, and transitional epithelial cells.[201] It can be assumed that TrxR levels in rat kidney and liver are comparable.[174,225] Kidneys contain large amounts of (mainly protein bound) selenium and 50% thereof is not associated with glutathione peroxidase. Jamba et al. present an autoradiograph from a ^{75}Se-labeled mouse kidney extract (Fig. 2 in Ref. 226) which shows two 58 kDa bands in different fractions—most likely mTrxR-1[47] and mitochondrial mTrxR-2.

O. Prostate, Testis, Ovary, and Uterus

In the rat female genital system, an intense immunostaining for both proteins is reported for follicular cells, early luteal and *theca interna* cells as well as for the germinal epithelium. Oocytes, atretic follicles, and old luteal cells were essentially negative.[161] The uterus and Fallopian tube showed a moderate staining. The male genital system was intensely stained for thioredoxin (but not for TrxR) in Leydig's interstitial cells of the testis and some spermatogonia. Thioredoxin reductase was found in spermatocytes, spermatides, and Sertoli cells. The prostates, seminal vesicles, and epididymis's epithelium were moderately to intensily stained for both proteins.[161] In testis a tissue specific selenoenzyme, closely related to TrxR—in fact initially assumed to be a pure TrxR (''hTR2'')[104]—now referred to as thioredoxin-glutathione reductase (TGR)[105] is present.

High TrxR–mRNA levels were reported in human prostate, testis, and—in contrast to the protein data gathered with the rat tissue—in the uterus. Trx–mRNA levels were considerably lower in these tissues.[195]

Two testis specific proteins with a CGPC-thioredoxin motif, which have therefore been named *sperm specific Trx* (Sptrx), have recently been identified: hSptrx-1,[178] a cytosolic protein, exhibits Trx activity, yet it appears that its cellular function is to act as an oxidant.[80,81] Its expression is restricted to the postmeiotic phase of spermatogenesis. As oxidation processes are important steps during spermatogenesis is speculated to play an essential role for the development of the sperm. hSptrx-1 (53 kDa) is far larger than typical thioredoxins and furthermore forms oligomers. It contains several putative phosphorylation sites which supports the concept of a regulated or regulating factor in spermatogenesis, particular in tail formation.[227]

hSptrx-2, present in the cytosol and nucleus and expressed from the pachytene stages onwards, is apparently monomeric.[179] Apart form its Trx domain containing the classical CGPC-motif, the protein possesses 3 putative NDP kinase domains. However, no catalytic function, neither classical Trx redox activity nor NDP kinase activity could be demonstrated. Thus its function remains speculative. It is argued that it might be a substrate of TGR. This would explain the presence of this tissue specific reductase. However, activity of the recombinant protein may have also been missed as regulatory phosphorylation did not occur in the heterologous expression system, which could have prevented the formation of the correct folding required for activity. Its low similarity (approx. 25%[179])

with other thioredoxins (including Sptrx-1) raises the question if this protein should in fact be subsumed to the thioredoxins if subsequent studies should also fail to demonstrate thioredoxin-like activity.

In one clinical case report, autoantibodies directed against thioredoxin reductase were reported in a patient with ovary adenocarcinoma.[228] However, this study used the *E. coli* TrxR for affinity purification, thus it is likely that the patient had developed antibodies after a bacterial infection and not—as claimed—*auto* antibodies. Nevertheless, it is well possible, that autoantibodies against Trx or TrxR are much more common than previously thought. If this should turn out to be correct, it may serve as a new parameter for diagnosis and follow-up in cancer patients.

P. Nervous Tissue

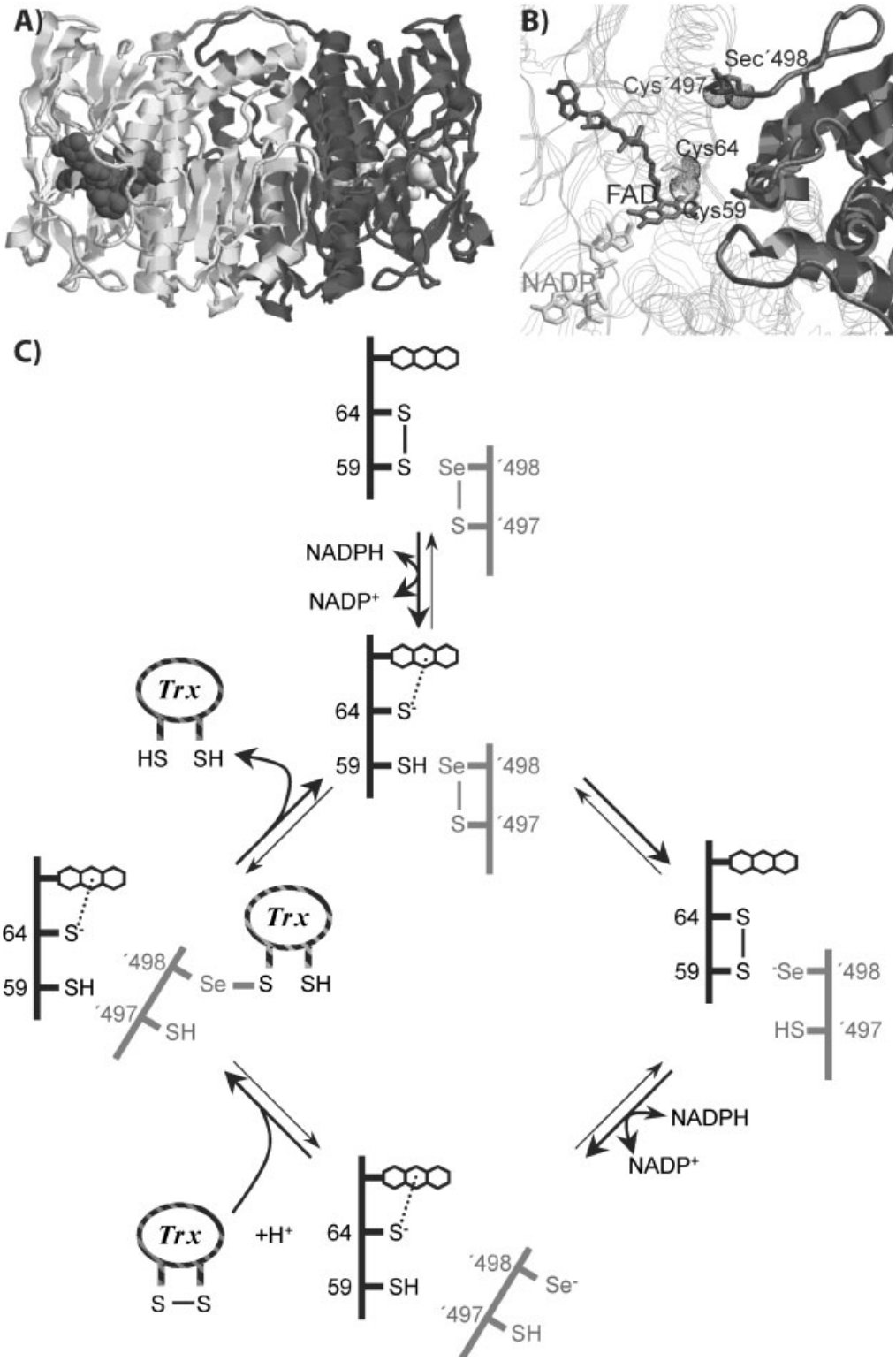
In the rat, large nerve cells of the central as well as of the autonomous and peripherial nervous system showed moderate to intense staining for Trx and TrxR as did most neuroendocrine cells, the choroid plexus epithelial cells, ependymal cells, and retinal pigment cells. Glia and stromal cells were essentially negative.[161] Significant concentrations of Trx (and glutaredoxin) are found in the hypophysis.[229] Data given by Hill et al.[174] indicates that TrxR-activity in the brain is protected even in states of selenium deficiency. Within the neuron, the thioredoxin system has been implicated in microtubule assembly.[230,231]

6. BIOCHEMICAL PROPERTIES

A. Catalytic Mechanism and Substrate Specificity

The substrate spectra of large and small TrxRs differ enormously. Whereas the small TrxRs exhibit a very narrow spectrum with thioredoxin being almost the sole substrate, large TrxRs stand out for their ''omnivorous'' behavior—with the selenium dependent isoenzymes at the top. Small, non-protein molecules such as 5,5′dithiobis-(2-nitrobenzoate),[232] alloxan,[33] dehydroascorbate,[107] selenodiglutathione,[37,233] ebselen,[234] *S*-nitrosoglutathione,[38] alkylhydroperoxides, methylseleninate[100] are as well substrates as are proteins like thioredoxin,[34] NK-lysin,[192] protein disulfide isomerase,[35] plasma

Figure 7. The catalytic mechanism of human and other large TrxRs. **A**: A computer model of homodimeric mammalian thioredoxin reductase. The protein backbone is shown as a ribbon, each subunit in a different color. The two bound FAD molecules are shown as space filling models. **B**: One catalytic reaction center is formed by two subunits. The backbone of one is shown as strands, whereas the backbone of the opposite subunit is shown in ribbon representation. Bound FAD (grey) and $NADP^+$ (light grey) are represented as stick-models. The flavin near the N-terminal redox active site (Cys59 and Cys64) is provided by one subunit, and the C-terminal redox active site of the same reaction center by the other subunit (Cys′497 and Sec′498). This explains, why only the homodimer is catalytically active. Each dimer forms two independent catalytically active centers. The sulfur and selenium atoms of the N-terminal cysteines and of the reduced C-terminal Cys and Sec residues are indicated by their dotted van-der-Waals-radii. This representation is based on the crystal structure of U498C c-rTrxR[328] (PDB-ID: 1H6V) using RasWin V2.7.1.1 for visualization. **C**: Sketch of the postulated mechanism for Trx reduction by large TrxRs.[235,236] Only one reaction center, yet formed by both subunits (indicated by black and grey lines), is shown. The oxidized enzyme (E_{ox}), due to the high NADPH:$NADP^+$-ratio is presumably a rare species under *in vivo* condition, can be reduced to an EH_2 species by NADPH.[32] The N-terminal redox active site exchanges the electrons with the C-terminal redox active site of the opposite subunit (see Refs. 351 and 353). Additional reducing equivalents provided by NADPH are taken up to yield an EH_4-species (Ref. 32 and S. Gromer, L.D. Arscott, C. H. Williams, Jr., unpublished results). The previously reported potential uptake of two further electrons (to a total of 6) as indicated by a dithionate titration[32,39] is presumably an artefact (C. Böhme, L.D. Arscott, and C.H. Williams, Jr., personal communication). Selective digest experiments suggest that the reduced C-terminal tail now moves to a more solvent exposed position.[48,235] Oxidized thioredoxin reacts with the reduced C-terminal tail's selenolate to yield a mixed selenenylsulfide, which is cleaved by the adjacent thiol (Ref. 351), to yield reduced thioredoxin and the initial TrxR-EH_2-species. Steady-state kinetics demonstrated an overall ping-pong mechanism as inidicated by this model.[57] It should, however, be pointed out, that a new molecule of NADPH might bind prior to the end of the oxidative half reaction, as suggested by pre-steady state experiments with the human enzyme (L.D. Arscott, S. Gromer, C.H. Williams, Jr., unpublished results).


A)
B)
Sec´498
Cys´497
Cys64
FAD
Cys59
NADP
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+H+
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Figure 7.

glutathione peroxidase,[191] calcium-binding proteins 1 & 2,[82] and many others. Even though there are minor differences within this list between the different large TrxRs and even though the physiological importance of many of these reactions is unproven a hypothesis for the catalytic mechanism must take them into account.

In collaboration with others we outlined and published a now widely accepted hypothesis for the catalytic mechanism of human thioredoxin reductase (Ref. 235 and Fig. 7) which is based on pre-[32] and steady-state kinetic.[57,235] This hypothesis should in its principles be applicable to all large TrxRs. Recent findings (Gromer et al.[351] and Bauer et al.[353]) further refined this initial model.

A key feature of the proposed mechanism of large TrxRs is the C-terminal relatively flexible tail which is responsible for the transport of electrons from the buried redox-center near the flavin to bulky substrates at the surface. The more exposed position of the reduced C-terminus enables a broader range of substrates to be reduced, but small molecules can also (although generally less efficiently) take over reducing equivalents from the N-terminal active site. A blueprint of the course of events for both types of TrxRs is shown in Figure 7.

The key intermediates of proposed mechanism (Fig. 7), especially the selenenylsulfide-bridge have meanwhile been confirmed by Zhong et al.[236] and Lee et al.[54]

The catalytic mechanism of the small TrxR is already known in atomic detail and shows a lot of differences to its large counterpart, the main feature being a domain rotation of 66° does not occur in large TrxRs (Fig. 8).

It should be pointed out, that either of the catalytic mechanisms of the two classes of TrxR—small and large—as well as some of the involved essential structures, in particular, the C-terminal sequence of the large TrxRs appear to be rather unique not only between species, but also within the same organism. Thus the development of mechanism-based drug-design is a promising approach, as it appears that the isoenzymes of, for example, host and pathogen can be chemically and therefore therapeutically differentiated.

7. MEDICAL ASPECTS

The thioredoxin system is involved in numerous cellular functions with potential medical applications. We would like to discuss some of these below.

A. Infectious Diseases

Infectious diseases are caused by transmittable pathogens such bacteria, fungi, parasites, viruses, and prions. Apart from prions possibly they all face the lethal armory of the host's immune defense system: reactive oxygen species formed by macrophages, pore forming immune toxins, and inactivating immunglobulines to name a few. To survive in this hostile environment, the pathogen needs effective defense mechanisms. As it is the survival of the species and not of the individual that is of importance in the long run, a rapid multiplication of pathogens is normally also of importance.

As already pointed out the thioredoxin system, if present, is a key player in the antioxidative system of the cell and may thus be regarded as part of the first line of defense against the immune system's respiratory burst as well as metabolism's ROS-byproducts. At least *in vitro* thioredoxins are capable of reducing immunoglobulins which leads to their inactivation.[237] Not all thioredoxins can inactive all subtypes of immunoglobulins nor is it proven that secreted Trx is in fact capable to reduce and thereby inactivate immunoglobulines *in vivo* to a relevant degree. There are, however, clinical and pathological findings such as the virtual absence of immunocomplex deposits on *Helicobacter pylori*—that could be explained by this proposed mechanism.[97] Even though there are reports that bacteria lacking thioredoxin and glutaredoxin can survive and multiply *in vitro*, it must be

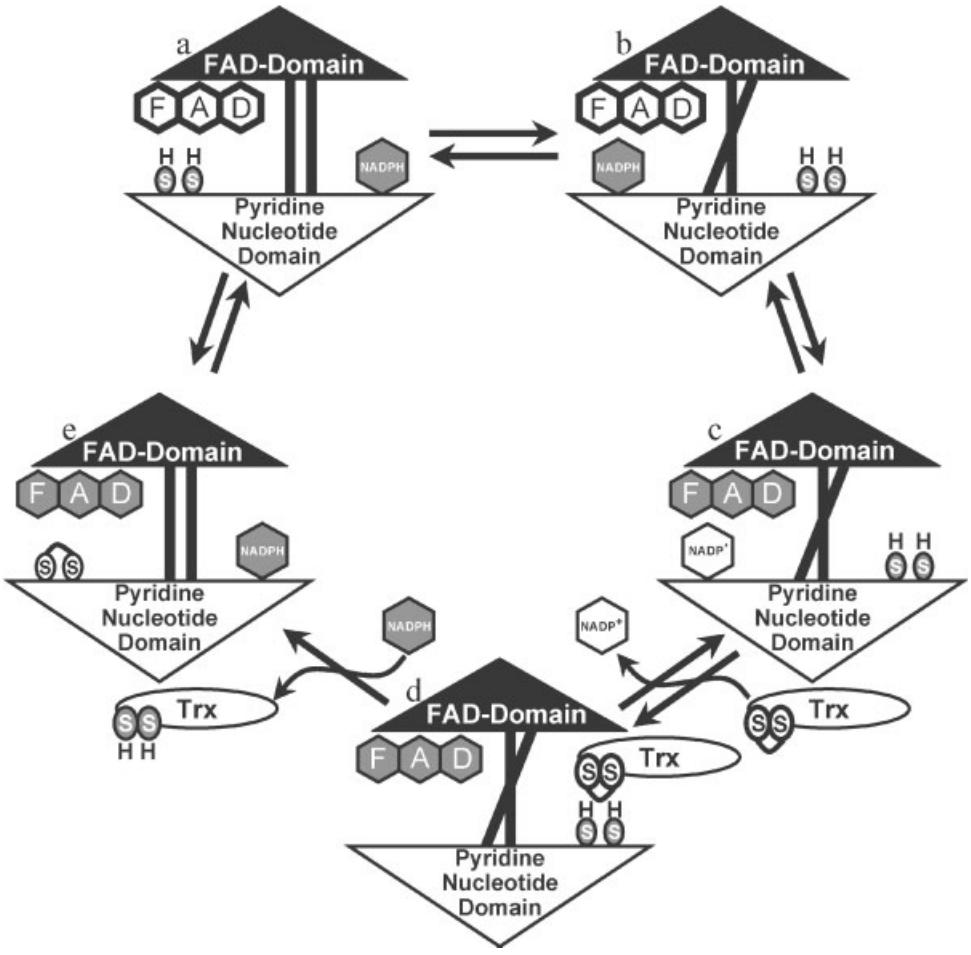


Figure 8. The catalytic mechanism of *E. coli* TrxR (derived from data presented in Refs. 345–347). Grey shaded structures represent transiently reduced states. The enzyme cycles between a two electron reduced state (EH_2) and a four electron reduced (EH_4) state during the catalysis.

pointed out that the growth rate was reduced and the bacteria required a nutritious medium for survival.[238] Thus it remains tempting to speculate that specific inhibitors of small TrxRs might serve as novel and effective antibiotics which, because of the significant differences to human isoenzymes, should exhibit only few side-effects. In fact, competitive inhibitors of *E. coli* TrxR, azelaic acid (Fig. 17B) and related compounds, are known to posses bacteriostatic effects.[239] It should be pointed out that these compounds were published as inhibitors of the *human* enzyme, yet most of the study was in fact done using *E. coli* TrxR as a substitute.[240,241] Using the enzyme purified from human placenta—we did not observe a significant inhibition up to 10 mM—a concentration that implies that contaminations of the ''inhibitor'' (assuming a 99% purity) may be already present in micromolar concentrations. This finding once again may highlight the importance to carefully review and interpret data gathered with a different system. However, it also supports the concept of a selective antibiotic since the compound does *not* inhibit the human enzyme in therapeutic concentrations. Similar considerations can be made for fungi which also express small TrxRs. Due to its socioeconomic importance, we would like to point out one variant of small TrxRs: *Mycobacterium leprae* has fused thioredoxin with thioredoxin reductase on the gene and protein level.[242–244]

This particular composition may serve as a new starting point in the search for new drugs again leprosy.

The species differences at the C-terminus of large TrxRs might serve as novel targets for antiparasitic drugs. For malaria, it was shown by Müller and coworkers that the *falciparum* thioredoxin reductase is essential for the *Plasmodium* parasite at least in the erythrocytic stages of its complex life-cycle.[245] A similar dependence was found in *Schistosoma mansoni* and its large TrxR-related thioredoxin–glutaredoxin reductase.[106]

Many important (especially so called tropical) diseases like malaria are vector born. As TrxR is apparently essential for many of these insects[95] and structurally different from the human enzyme it appears promising to develop TrxR-inhibitors for the vector's enzyme (Refs. 60 and 352). Caution must, however, be taken, as it is very likely that TrxRs of many insects are similar, and thus unselective spraying of the environment with such inhibitory compounds to eliminate the vector of a disease may result in unexpected and deleterious effects on many ecosystems. For a more detailed review on the thioredoxin system of parasites (see Ref. 10).

A number of viral diseases are associated with increased plasma thioredoxin levels which is regarded a marker of oxidative stress. Epstein–Barr virus (EBV) and human T-lymphotropic virus type I (HTLV-1) are the best known examples, yet we will deal with them in a later section. In one report hepatitis C virus (HCV) related diseases—asymptomatic carriers, chronic hepatitis as well as liver-cirrhosis and hepatocellular carcinoma—were associated with significantly increased serum levels of thioredoxin.[246] One of the escape mechanisms of the hepatitis C virus leading to its high rate of chronicity ($>70\%$) is its high mutation rate which is assumed to be mainly due to the high error rate of the viral RNA-polymerase.[247] However, in the above cited article by Sumida et al.,[246] it was further noted that serum Trx levels correlated inversely with the results of interferon treatment. It is thus tempting to speculate that an increased Trx serum level reflects an increased cellular level of oxidative stress resulting in a further increased mutational rate of the HCV-genome in these patients but also of the host cell's DNA. This may eventually result in hepatocellular carcinoma (HCC). It must, however, be stated, that others did not find increased levels of serum thioredoxin[248] in patients suffering from HCV-related liver disease other than hepatocellular carcinoma. In fact, Trx-levels do decrease after the surgical removal of the tumor. Apart from its potential diagnostic value,[249,250] these findings need confirmation as they may have significant impact on therapy as well: animal models with chemically induced neoplastic liver nodules indicate that cytosolic TrxR activity increases (350%) whereas mitochondrial TrxR activity decreases by approx. 60% in comparison to healthy liver tissue.[251] The net result is an increased cellular resistance against the immune system's oxidative armory and facilitated DNA-synthesis due to the increase in cytosolic TrxR activity. The tumor's benefit, if any, from the decrease in mitochondrial TrxR activity remains unclear, as it is assumed that higher activities are protective against apoptosis. Elevated mitochondrial hTrx-2 levels are reported to be protective against etoposide (VP-16) toxicity.[153] The observed decrease in mitochondrial TrxR activity might thus be the biochemical basis for the clinically observed superiority of the combinational therapy of etoposide and adriamycin.[252] These findings do, however, result in a relevant clinical problem: if serum thioredoxin levels mirror cellular oxidative stress it is reasonable to treat preneoplastic lesions (e.g., asymptomatic HCV carriers, acute and chronic hepatitis and liver cirrhosis) with selenium and other antioxidants, yet this therapy may bounce as soon as the disease has progressed to a true neoplastic lesion. Clinical data do, however, also suggest that selenium supplements are also beneficial in overt tumors, indicating that other mechanisms are involved as well.[253,254]

Also HIV-infected individuals show increased serum thioredoxin levels[255,256] and these levels correlate well with stage and progression of the disease. Full-length hTrx-1 does suppress HIV-production.[257] Macrophages, however, are capable of converting thioredoxin into the C-terminally truncated $\sim$10 kDa protein called "eosinophil cytotoxicity-enhancing factor ($=$ECEF)" which in contrast enhances HIV-production.[257] As the determination of "thioredoxin" in the studies by

Nakamura et al.[255] were done using monoclonal antibodies directed against specific epitopes of the Trx-protein it may be argued that ECEF was misinterpreted to some extent as its precursor Trx in HIV-infected patients. Recent reports suggest, that ECEF is identical with Trx80,[79] which has also been reported to be present in the plasma of healthy individuals.[79]

In contrast to serum levels an earlier publication on lymph node biopsies of HIV-patients reports a significant loss of thioredoxin-production in this tissue.[258]

The interpretation that HIV is accompanied—especially during the later stages of the disease—by a significant burden of oxidative stress is supported by many studies.[259–262] This may be at least in part due to the decreased synthesis of the antioxidative selenoproteins glutathione peroxidase[263] and TrxR and an increase of low molecular weight Se-compounds.[264] The precise mechanism for this change remains unclear, yet it is noteworthy that selenium supplements appear to be clinically beneficial.[265] If we assume that it is the conversion of secreted Trxinto ECEF/Trx80 enhances the progress of the disease, the clinical consequences should be to counteract oxidative stress by antioxidants and to inhibit the conversion of Trx into ECEF/Trx80 (Fig. 9). If this is possible a co-treatment with recombinant thioredoxin may become beneficial, too. However, as the presence of a truncated Trx (Trx80/ECEF) was recently reported in the plasma of healthy individuals,[79] it may well be that it serves essential physiological functions.

The thioredoxin system was linked to prion diseases in one report.[266] Prion diseases like bovine spongiform encephalopathy (BSE), widely known as mad cow disease, and its human counterpart Creutzfeld-Jacob-syndrome (CJS) are most likely caused by a different conformation (PrP^{Sc}) of a cellular protein (PrP^{c}) which leads to PrP^{Sc}-precipitation and consecutive cell death (for a review see Ref. 267). It is known that the ''normal'' conformation can be precipitated using reducing agents such as dithioerythritol (= DTE). *In vitro*, the thioredoxin system is capable to perform this task much faster. However, the resulting structure does not show proteinase K resistance as does the ''naturally'' converted protein. Furthermore, the conformational change from PrP^{c} to PrP^{Sc} is believed to take place in the endoplasmatic reticulum. The data on the presence or absence of a functional thioredoxin system with access to this compartment are, however, contradictory, despite the recent discovery of ER-proteins with thioredoxin related C*XX*C-motifs.[84] Treatment of infected animals with effective TrxR and/or Trx-inhibitors—which should result in some protection—might provide further insight into this interesting, yet highly speculative link of the Trx-system.

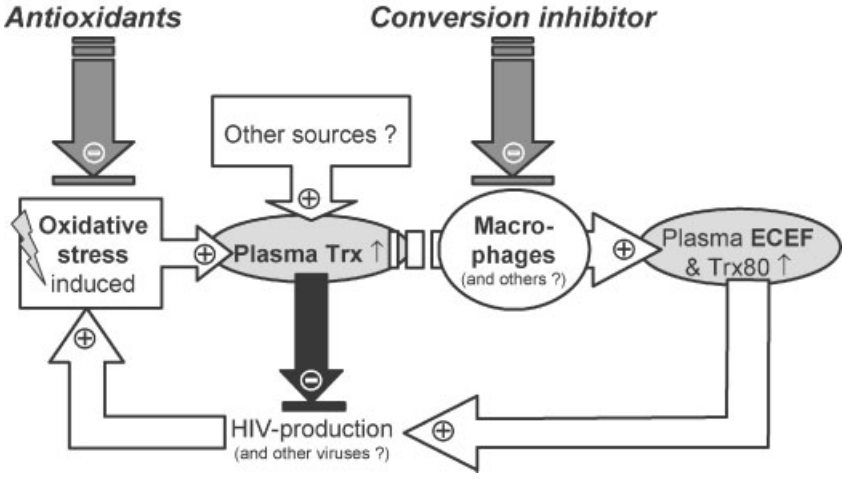


Figure 9. Scheme of the interaction of thioredoxin, ECEF/Trx80 and HIV-production based upon Refs. 257, 348. Also indicated are theoretical therapeutic approaches. White arrows indicate stimulation, dark arrows inhibition.

B. Non-Infectious Diseases

1. Neoplastic Diseases

The sequence of events in the course of a tumor is often divided into five stages—initiation or transformation, progression, local invasion, distant metastasis, and finally resistance to therapy. In all stages, the thioredoxin system may play a role. During initiation, the cell is damaged on the DNA level in multiple ways leading to an increased product of cell-division rate and cellular lifespan. Many carcinogens act either directly or indirectly via the formation of radicals such as reactive oxygen species. Thus, the antioxidative properties of the thioredoxin system should be able to reduce the burden of these potentially deleterious agents. The fact that in clinical trials selenium supplementation led to a reduction of several tumor entities incidence rates[224] may be regarded as indirect evidence for this proposition: selenium supplementation increases glutathione peroxidase and thioredoxin reductase activities which in turn leads to the formation and maintenance of methylselenol (Fig. 10)—a compound considered to be a key metabolite in the tumor-preventive effects of selenium.[172,268,269] Methylselenol is a small compound which can easily penetrate membranes and react with ROS and thereby detoxify them. The oxidized product—either methylseleninate or methylselenenate—are easily reduced by thioredoxin reductase to methylselenol in the methylselenol-cycle (Fig. 10).[100] Thus, methylselenol is considered to be capable of clearing membranes from potentially harmful agents also in areas that are inaccessible to charged antioxidants like GSH.

It must, however, be pointed out that the dose–effect-relationship of selenium is not completely linear and apparently much more complex as—at least *in vitro*—very low selenium supplements have been shown to increase cell growth rates.[270]

During growth and progression phase, the before beneficial effects of the thioredoxin system may change to its opposite. Being responsible for providing reducing equivalents to ribonucleotide reductase, it is involved in a central process of cell division. As pointed out earlier, other systems can substitute for the thioredoxin system at least *in vitro* yet it is typical for all proliferating tissues to show increased TrxR and Trx content in immunostaining when compared to resting tissue.[161] It is therefore not surprising that almost all tumors studied so far exhibit several fold increased TrxR- and Trx-levels.[152,217,218] The thioredoxin system furthermore protects the transformed cell from attacks of the immune system as it detoxifies, e.g., hydrogen peroxide and several nitric oxide

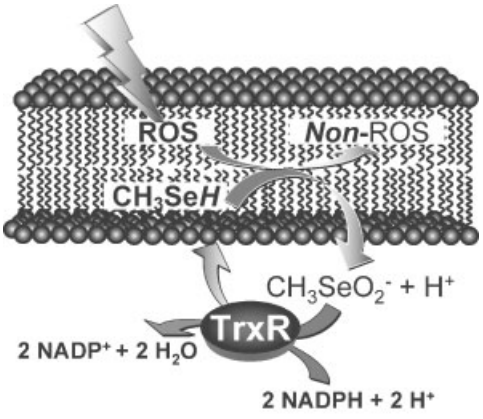


Figure 10. Methylselenol-cycle. Reactive oxygen species (ROS), formed for example in or near the plasma membrane are detoxified to nonreactive products (*non*-ROS) by methylselenol. The resulting oxidation products, methylselenenate and methylseleninate are charged and thus expelled from the membrane to the cytosol. Here methylselenol is regenerated by thioredoxin reductase (or by reduced glutathione) thereby completing the methylselenol-cycle.

species.[38,271] It has also been shown that mammalian TrxR and Trx can inactivate NK-lysin—a pore-forming peptide in the armory of natural killer cells.[192,272] The *in vivo* significance of this last finding depends on the presence of active TrxR in the plasma membrane or in the extracellular fluid with the need for a supply of reducing equivalents by (most likely) NADPH. Even though TrxR has been described as membrane associated[188] and even as a product that might be secreted by cells, it has never been shown that there is a direct interaction between intracellular reducing equivalents, TrxR and extracellular substrates. It has been argued that reduced Trx, which is known to be secreted, may perform this reducing task. Taking the energy required to produce and secrete Trx into account, this sounds unlikely if the sole purpose was to provide two reducing equivalents, thus one may speculate that the secreted Trx must be kept in a reduced state by a (functional) TrxR in order be have significant impact on compounds such as NK-lysin. Apart from a reduction by a TrxR in the plasma membrane whose cytosolic domain would obtain its electrons from NADPH,[273] one could think of a reuptake-mechanism. A Trx reuptake is known, yet it remains unclear if the protein stays intact in this process.[274]

Thioredoxin and truncated Trx secretion are common features of normal and especially neoplastic cells. Acting as mitogenic cytokines they can promote tumor formation and propagation. This is of importance not only in rare cases but in quite common diseases such as B- and T-cell leukemias.[275,276]

Thus the thioredoxin system may influence virtually all four phases of tumorgenesis via its involvement in transcription and translation.

Furthermore, the thioredoxin system may be involved in a serious clinical problem: drug resistance. As many drugs directly or indirectly induce apoptosis, one must recall that many functions of the thioredoxin system are antiapoptotic. Thus, it is not unexpected that many highly chemotherapy resistant tumors express high levels of TrxR and Trx.[275–280] Thioredoxin system inhibitors should be capable to overcome or at least ameliorate this serious medical problem.

2. Non-Neoplastic Diseases

a. Rheumatoid arthritis and related diseases. To subsume rheumatoid arthritis and associated diseases here is somewhat arbitrary as the etiology of this common disease remains unresolved and some infectious agents—especially of viral origin (e.g., Epstein–Barr virus see Ref. 281)—are still prime candidates. Reasons to discuss it along with neoplastic diseases can be given too.

Sjögren's syndrome, a chronic systemic inflammatory disorder associated with EBV infection, is characterized by lymphocyte infiltration of mucosal and other tissues.[282] The patients suffer from dryness of the eyes, mouth, and other mucous membranes as well as from rheumatic symptoms. This syndrome shares features with rheumatoid arthritis (RA), a very common chronic disease characterized by symmetrical inflammation of peripheral joints; indeed the two conditions can appear together.[281] In the inflamed tissues of patients with Sjögren's syndrome, a strong correlation between virion production and Trx synthesis was observed.[282] The fact that the activity of IL-1, an inflammation mediator in rheumatoid arthritis, is enhanced by reduced Trx which itself is an effective growth factor also in lymphatic tissues supports the notion that the Trx-system is involved in the pathophysiology of chronic diseases.[163]

The potential involvement of TrxR in the pathogenesis of rheumatoid arthritis is underlined by recent *in vivo* data. Significantly increased levels of Trx and TrxR were found in synovial fluid and tissue—but not in blood plasma—of patients suffering from rheumatoid arthritis. This was not the case in individuals with other joint diseases such as gout or osteoarthritis.[283] In rheumatoid arthritis, the synovial Trx-levels correlated with the local severity of inflammation. This supports the idea to consider the Trx-system not only as a drug target for the treatment but also as a potential clinical parameter for diagnosis and therapeutic management since local alterations normally precede systemic symptoms.

Another indication is that organic gold compounds such as auranofin and aurothioglucose are widely and effectively used in the treatment of rheumatoid arthritis.[281] These compounds are known to be highly effective inhibitors of thioredoxin reductase and do act as such *in vivo* as shown, for example, in a mouse model.[284] The gold compounds are primarily accumulated in immune cells and lead to a reduced cytokine induced release of granule proteins in adherent neutrophils.[285] This selective accumulation may explain why ROS generation is decreased despite the inhibition of the antioxidative enzyme TrxR.

b. Arteriosclerosis. Results on the involvement of the thioredoxin system in the prevention but also in the development of arteriosclerosis are still limited and in part contradictory. A reason for this might be the finding that endothelial cells from different species and tissues do not show identical patterns of selenoenzymes.[286] Whereas assayed human cells were comparable, bovine and porcine cells were not. Human umbilical-vein endothelial cells (HUVEC) exhibit increased TrxR-levels after selenium supplements, which are known to protect endothelial cells from oxidative damage.[287] The thioredoxin system has, however, also been reported to be involved in the formation of the neointima in arteriosclerotic lesions.[288] It remains therefore unclear if the thioredoxin system is solely beneficial or whether conditions exists in which its action becomes harmful.

c. Reperfusion injury. Reperfusion injury is believed to be largely due to the formation of ROS in the early stages of reperfusion. Lung[289,290] and cardiac reperfusion injury[291] was found to be ameliorated by thioredoxin infusions. However, in the case of cardiac reperfusion injury it turned out that 100 nM thioredoxin was superior to 1 μM in preventing arrhythmias. Assuming—as proposed—a radical scavenging mode of action, this result is at least unexpected. So far there is no proven enzyme that is capable of efficiently reducing extracellular thioredoxin. 100 nM is, however, an extremely low concentration for a radical scavenger *per se* as we have to assume that it cannot be recycled. Thus the data presented so far are indicative, yet not convincing to us. The beneficial effects of Trx may, however, result from Trx acting as a signal molecule and not primarily as a radical scavenger. One more recent study indicates that the combination of a thiol donor, such as *N*-acetyl-cysteine and Trx, might turn out to be superior.[292]

In any case, it must be proven that the use of *i.v.* Trx is of such benefit that it justifies the high costs. Furthermore, one must take into account potential long-term side effects, e.g., on occult neoplastic cells.

8. INHIBITORS OF THIOREDOXIN AND THIOREDOXIN REDUCTASE

Given the large number of processes the thioredoxin system influences, relatively few truly different inhibitors of the involved enzymes are known. Indeed far less than 200 entries are found today in Medline using the keywords ''thioredoxin inhibitor''; many thereof do not even deal with direct inhibition of Trx or TrxR. Unfortunately a number of inhibitors are not listed in Medline, reducing the access for the broad community. Technically speaking, care must be taken as many compounds are reduced by large TrxRs at the expense of NADPH. If their K_M-value is significantly lower than the K_M-value of the assay substrate (e.g., DTNB-reduction-assay: K_M for DTNB is around 500 μM) and NADPH-consumption is not measured directly compounds may easily be mistaken as inhibitors.[232,293] This may have supported, e.g., the assumption that ebselen is an inhibitor of human TrxR[294] whereas Arteel et al. identified ebselenoxide as a substrate of the enzyme.[234]

A. Inhibitors of Thioredoxin

Trx inhibitors are a rather recent concept and to our knowledge only a few selective compounds have so far been published as being effective. From a theoretical point of view, they may have some advantages in cases where solely extracellular Trx-activity but not TrxR plays a disease-promoting part.

1. Alkyl-2-Imidazolyl Disulfides and Related Compounds

These compounds were originally designed as TrxR inhibitors but during subsequent studies it turned out that some of them inactivated Trx at concentrations far below those needed for TrxR inhibition.[295–297] It is proposed that it is the time and concentration dependent irreversible thioalkylation of Cys73 of hTrx which leads to inactivation and is responsible for their growth inhibiting effect.[296]

In cell culture experiments, primary cells from an acute T-cell lymphoblastic leukemia were effectively treated with 1-methylpropyl-2-imidazolyl disulfide (Fig. 11A).[275]

2. Naphthoquinone Spiroketal Derivatives

A series of naphthoquinone spiroketal compounds has been synthesized and tested for their inhibitory effects on the thioredoxin system.[298] Some compounds preferentially inhibited TrxR, others Trx. The IC_{50}-values for many of these compounds are in the lower micromolar range. For the most potent one's, palmarumycin CP1 (Fig. 11B), it was only 350 nM when measuring Trx-inhibition. In cell growth inhibition assays, these compounds were effective as well, but even many of those compounds that did not inactivate the thioredoxin-system turned out to be valuable cell growth inhibitors. It is therefore reasonable to assume that this family of inhibitors has at least one further cellular target.

Related to this group is pleurotin. It's IC_{50}-value for the thioredoxin system has been reported to be 170 nM.[299]

B. Inhibitors of Thioredoxin Reductase

Most irreversible inhibitors of thioredoxin reductase act apparently via a reaction with one or more redox-active residues (Cys and Sec) as they do not affect the enzyme in the absence of NADPH in most cases indicated. Thus electrophilic agents are candidates for a potential inhibitory effect.

1. Nitrosoureas

Nitrosoureas, such as the clinically still widely used carmustine, are non-selective carbamoylating or alkylating agents that easily react with thiols and selenols but also other functional groups.[300] Most of these compounds are unstable in solution and rapidly disintegrate to form their reactive intermediates (Fig. 12B). Therefore, comparatively high—that is millimolar concentrations—are used for the treatment of brain tumors and certain lymphomas. One advantage of nitrosoureas is their capability to cross the blood–brain barrier.

All members of the glutathione reductase enzyme family in their reduced form are inhibited by nitrosoureas (see e.g., Refs. 300–302). TrxR is no exception to this rule. The first report on human TrxR inhibition was published by Schallreuter et al.[303] All tested nitrosoureas (Fig. 12A) effectively inhibited the NADPH-reduced enzyme. Similar results for the human placenta and the mouse enzyme were later published by our group.[32,47,210] However, Schallreuter et al. also claim that the inactivation by nitrosoureas was reversible by the addition of reduced thioredoxin (in the case of TrxR) or reduced

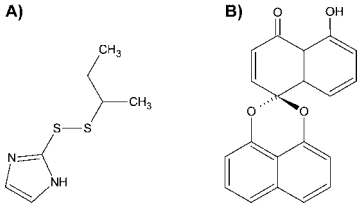


Figure 11. **A**: Structure of 1-methylpropyl-2-imidazolyl disulfide (also referred to as PX-12 or III-2). **B**: Palmarumycin CP_1. Both compounds can also inhibit TrxR, yet, they act mainly via the inhibition of Trx.

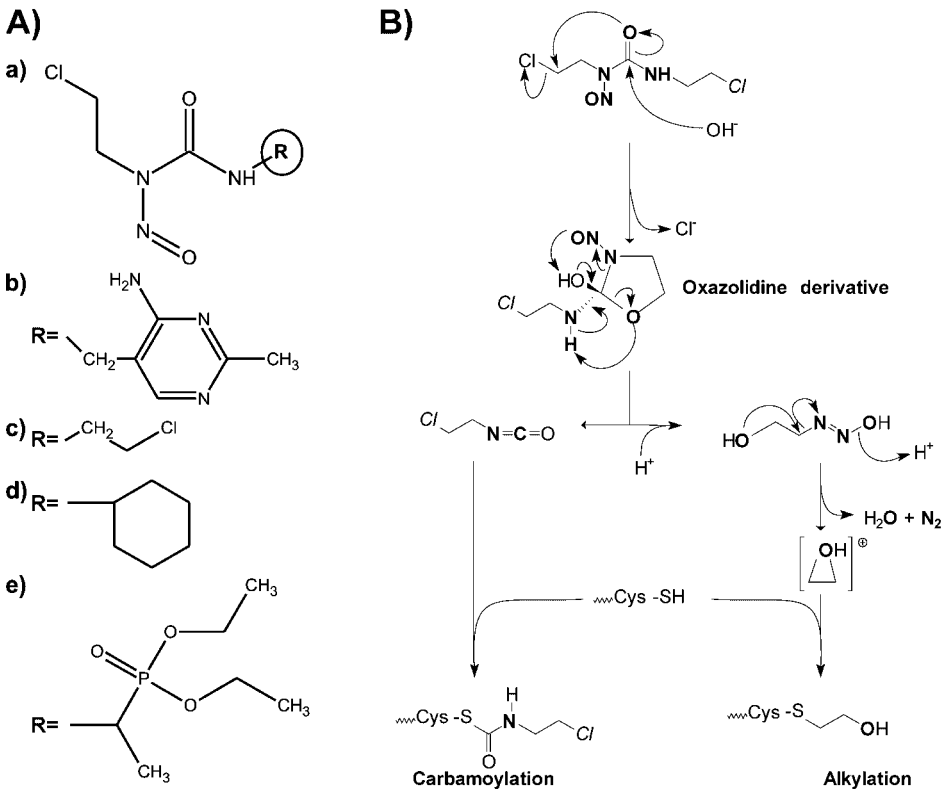


Figure 12. **A**: Nitrosoureas known to inhibit human TrxR[32,303]. The basic structure—1-(2-Chloroethyl)-*N*-nitrosourea—is given in (**a**). The different substituents for R in (a) of several nitrosoureas are given below: (**b**) 3-[(4-amino-2-methyl-5-pyrimidinyl)-methyl]-1-(2-chloroethyl)-*N*-nitrosourea (nimustine, ACNU); (**c**) 1,3-bis-(2-chloroethyl)-*N*-nitrosourea (carmustine, BCNU); (**d**) 1-(2-Chloroethyl)-3-cyclohexyl-*N*-nitrosourea (lomustine, CCNU); (e) Fotemustine. **B**: Formation of reactive carmustine (BCNU) intermediates and their reactivity with an enzymic cysteine. The formation of the alkylating intermediate is considered to be slower, thus carbamoylation is favored (left hand branch). In the case of 1-(2-chlorethyl)-3-hydroxyethyl-*N*-nitrosourea (HeCNU; right hand branch), in which one of the chlorine atoms (here shown in italics) is replaced by a hydroxyl group, an intramolecular carbamoylation takes place, favouring the alkylating reaction of the remaining intermediate.[301,349]

glutathione (in the case of glutathione reductase). At least for glutathione reductase this finding was not reproducible in our hands *in vitro*,[k] nor did DTE reactivate the nitrosourea inactivated TrxR[l].

Nitrosoureas are—as pointed out before—unselective and difficult to handle. We therefore do not recommend them for routine TrxR inhibition. Their effect on TrxR must, however, be taken into account when they are used for other purposes.

2. Gold Compounds

Gold containing formulations have been used in medicine for ages. At the end of the 19th century, heavy metal compounds gained considerable interest as chemotherapeutic agents.[305] Drugs with a proven effect are the antirheumatic gold(I)-compounds (Fig. 13), even though their mode of action is still a matter of debate. The interaction of gold(I) with selenoenzymes was first addressed by Chaudière and Tappel[306] who investigated the effects of aurothioglucose on glutathione peroxidase and by Berry et al.[307] who studied its effect on deiodinase I.

[k]Babson and Reed[304] do, however, report a slow reactivation of glutathione reductase in erythrocytes after nitrosourea treatment.

[l]The carbamolyating modification of glutathione reductase can be lost if the enzyme is fully denatured. This technique does, however, also lead to a loss of enzymatic activity.

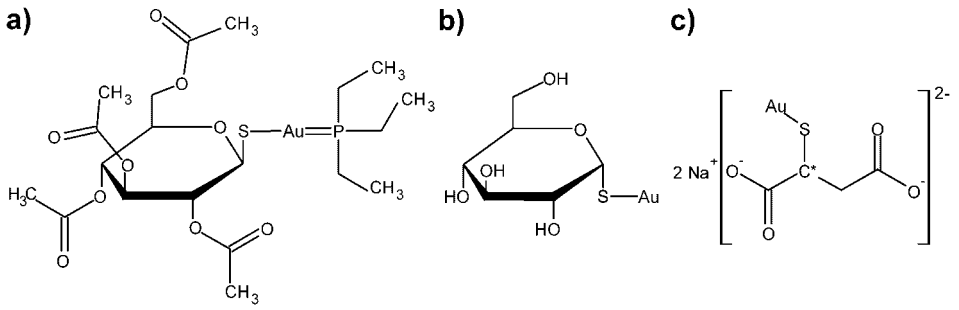


Figure 13. Several organic gold compounds, clinically commonly applied as antirheumatic drugs, are effective inhibitors of mammalian TrxR *in vitro*[57,155,174,308] and *in vivo*.[204,284] Shown are the structures of (**a**) auranofin (Ridaura®); (**b**) aurothioglucose (Solganol®); and (**c**) disodium aurothiomalate (Myocrisin®). Other selenoenzymes have been shown to be inhibited by these compounds as well,[306,350] whereas the Cys-containing enzymes are by orders of magnitude less susceptible, indicating a selectivity for selenolates. For TrxR inhibition, almost only stoichiometric concentrations are required, rendering this enzyme a prime candidate for the still not resolved mode of action of these drugs.[57]

Kristina E. Hill was the first to study the effect of aurothioglucose on rat TrxR in crude extracts[174] and actually developed an TrxR-tissue assay based upon her results.[308] At the same time, we studied aurothioglucose and auranofin as inhibitors of purified hTrxR-1.[57] More recently, aurothiomalate was also identified as an inhibitor of mammalian TrxR by Smith et al.[204]

Thioredoxin reductase is far more susceptible toward inhibition by gold compounds than glutathione peroxidase or glutathione reductase. Auranofin acts upon TrxR in almost stoichiometric concentrations ($K_i = 4$ nM). Glutathione reductase and glutathione peroxidase are inhibited in the micromolar range, that is at 1,000-fold higher concentrations. We have thus modified the original TrxR-tissue assay by Hill et al. using auranofin, as this compound is easier to handle and more selective than aurothioglucose.[293]

Gold(I) complexes and especially auranofin are among the most effective and selective inhibitors of mammalian TrxRs known today. One further advantage of auranofin is the fact that it is commercially available, well soluble, and relatively stable in DMSO.

Rigobello et al. reported that auranofin is also capable of inhibiting mitochondrial rTrxR-2,[155] which results in mitochondrial swelling and apoptosis. These findings are consistent with an increased mitochondrial membrane potential under hTrx-2 overexpression as reported by Damdimopoulos et al.[153]

Auranofin has been evaluated in screening trials as a potential antineoplastic agent as early as 1981.[309] It proved at least as effective as 5-fluorouracil and cisplatin, both commonly used cytostatic drugs. At that time, the inhibitory effects of auranofin on TrxR were of course not known. It was, however, stated, that DNA-synthesis decreased rapidly under auranofin treatment. The used amount of auranofin was 6 mg/kg body weight. Assuming an equal body distribution a resulting concentration of approx. 10 μM can be calculated. Taking our results into account, it is unlikely that the resulting inhibition of glutathione reductase ($<40\%$ at this concentration) is responsible for the effect. One may thus regard the effects of auranofin on DNA-synthesis[309] as supporting evidence for the importance of TrxR in the synthesis of desoxyribonucleotides in mammals. However, others could not reproduce the effectiveness of auranofin on other cells[310] and also reported a non-selective inhibition of DNA, RNA, and protein synthesis caused by auranofin.

3. Platinum Complexes

Cis–platinum(II)-complexes are well known antineoplastic compounds. Two compounds—*cis*-diamminedichloroplatinum(II) (CDDP, cisplatin, Fig. 14a) and *cis*-diammine-(1,1-cyclobutanedicarboxylato)platinum(II) (CBDCA, carboplatin, Fig. 14b)—are clinically widely used as

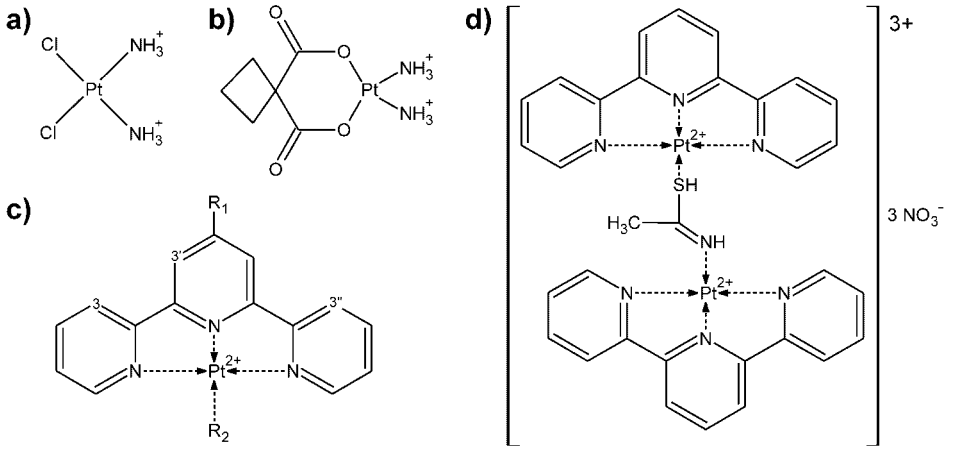


Figure 14. Structures of several platinum(II) complexes. **a**: Cisplatin (CDDP); (**b**) carboplatin; (**c**) basic structure of (2:2′:6′:2′′-terpyridine)platinum(II) complexes; (**d**) *N,S*-bis(2,2′:6′,2′′-terpyridine)platinum(II) thioacetimine trinitrate. Particularly the terpyridine–platinum(II)-complexes are excellent inhibitors of TrxR[316], acting in stoichiometric concentrations. Cisplatin (a) and its *in vivo* metabolite—the glutathione adduct bis-(glutathionato)-platinum(II)—are inhibitors of the thioredoxin system as well.[312] Interestingly, carboplatin (b)—even though closely related to cisplatin—is reported not to be an inhibitor under similar conditions.[312]

effective drugs in a large number of chemotherapeutic regimes. Their major mode of action is considered to be due to direct DNA-damage. Cisplatin is known as a potent electrophile that readily reacts with thiols, selenols, and other nucleophiles available. It is therefore not surprising that *cis*-platin and *trans*-platin are effective inhibitor of mammalian TrxR under reducing conditions.[311,312] Interestingly, carboplatin did not show significant inhibition.[312] The major cellular metabolite of cisplatin, its glutathione adduct, was also capable of inhibiting the thioredoxin system. Cisplatin- (and CDNB-)modified hTrxR-1 as well as a selenium-deficient hTrxR-1 have been shown to efficiently induce apoptosis.[313]

A series of new platinum(II) complexes has been synthesized recently. The general structure of these (2:2′:6′:2′′-terpyridine)platinum(II) complexes is known for its DNA-intercalating properties[314] and for the inhibition of *Trypanosoma cruzi* trypanothione reductase yet the closely related enzyme glutathione reductase was far less susceptible to inhibition.[315] These compounds are also highly effective inhibitors of human TrxRs and act at almost stoichiometric concentrations. This *in vitro* activity correlated well with their cytostatic effectiveness against different human glioblastoma and head-and-neck-squamous carcinoma cell lines. In extracts of these cells, the inhibition of TrxR was confirmed.[316]

Since these compounds attack two different targets within the metabolic pathway of DNA synthesis, they are highly interesting as this approach may reduce resistence formation and the need for high concentrations at once. Animal studies are underway and the data obtained so far are promising (Herold-Mende et al., personal communication).

4. Dinitrohalobenzenes

1-Chloro-2,4-dinitrobenzene (2,4-CDNB) was identified as a covalent inhibitor of mammalian thioredoxin reducase by Arnér et al. in 1995.[317] In fact, 2,4-CDNB does not only inhibit Trx-reduction but also induces a strong increase in the enzyme's NADPH-oxidase activity which leads to the formation of superoxide.[318] Thus, 2,4-CDNB converts the anti-oxidative enzyme TrxR into a pro-oxidative enzyme and therefore fulfills the criteria of a turncoat inhibitor.[319] The modification by the 2,4-dinitrophenyol group is easily detected in the absorption spectra of the enzyme[293] and Nordberg

et al. identified the C-terminal selenocysteine and its adjacent cysteine as the modified residues.[320] Interestingly, the isomers of 2,4-CDNB, namely 3,4-CDNB and 2,5-CDNB as well as the related compounds 1,4-dichlorobenzene or 4-chloronitrobenzene do not react with mammalian TrxR. The fluorine analog of 2,4-CDNB, 1-fluoro-2,4-dinitrobenzene, known as Sanger's reagent, inhibits the enzyme and also induces oxidase activity. The closely related enzyme glutathione reductase is attacked by 2,4-CDNB,[321] yet at a 100-fold slower rate than TrxR. This suggests that under *in vivo* conditions primarily TrxR (and not glutathione reductase) is inhibited. As 2,4-CDNB is used clinically, e.g., as a locally applied immunostimulating agent in the treatment of malignant melanoma (e.g., Ref. 322), its mode of action as well as its side effects must be discussed with respect to its inhibition of hTrxR.

5. Thiol Alkylating Agents

A number of research compounds including iodoacetic acid, iodoacetamide, 5-iodoacetamidofluorescein, and 4-vinylpyridine are widely used for the alkylation of thiols and presumably also selenols.

All these compounds have been shown to inhibit mammalian TrxR (see Refs. 104, 320 and authors' observation). It is interesting to note that the closely related enzyme glutathione reductase is inhibited by iodoacetamide but not by iodoacetic acid (L. Krauth-Siegel, personal communication and Ref. 323). *Drosophila* TrxR is C-terminally but not N-terminally modified by iodoacetamide.[353]

6. Organochalcogenides

Organotellurium and organoselenium compounds have been synthesized and screened for their inhibitory effects on mammalian TrxR.[294,324,325] Many of these compounds are efficient antioxidants. However, almost all the organotellurium compounds did very strongly and as judged from low glutathione reductase inhibition rates, very selectively inhibit human TrxR *in vitro*. Yet, data of cell culture experiments does not fully correlate with the non-competitive *in vitro* inhibition rates of TrxR, suggesting different modes of action within the cell or differences in bioavailability. The latter view is supported by significant differences in IC_{50}-values between the tested cell lines.[324,325]

As the most potent antioxidants in the diaryltellurium series turned out to be the most effective hTrxR inhibitors as well (Fig. 15), it was suggested that they act via the selenolate of TrxR after oxidation to the tetravalent state ($R_2{-}Te^{+II} \rightarrow R_2{-}Te^{+IV}O$) and subsequently lead to a subunit cross-linking.[324]

Even though not yet as effective as platinum(II)- or gold(I)- based drugs, tellurium compounds do certainly provide interesting lead structures for the development of new TrxR-inhibitors. However, in the presently tested compounds bioavailability and *in vivo* inhibitory effects are not yet ideally combined.

7. 10-Aryl-Isoalloxazines

Several FAD analogues impair the activity of human glutathione reductase. It was thus reasonable to test flavin analogues as inhibitors of glutathione reductase.[326] One series of these inhibitors, 10-arylisoalloxazines, turned out to be very effective. Unexpectedly, however, these compounds do not act as FAD analogues but in fact bind at the twofold axis between the subunits.[327]

As glutathione reductase and human thioredoxin reductase are structurally very similar,[328] it was considered possible that these compounds may also inactivate TrxR. In our experiments, however, none of the 28 compounds tested showed a significant inhibition at concentrations known to be effective for glutathione reductase.[210]

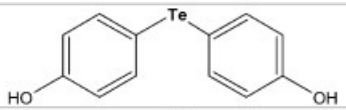
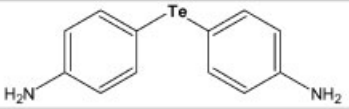
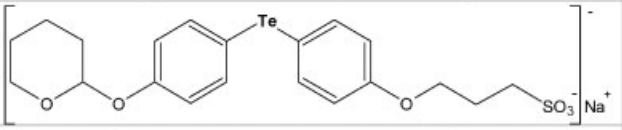
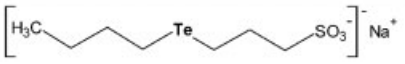
Tellurium compound structure	IC_{50} [μM] for hTrxR-1 inhibition in *in vitro* assays	IC_{50} [μM] for the inhibition of colony formation in cell culture studies		
		HT 29 cells	MCF 7 cells	Colo 320 cells
a) 	10.4	n.d.	1.9	3.8
b) 	2.3	n.d.	2.3	38.3
c) 	0.72	15.0	9.7	n.d.
d) 	0.6	> 30	> 30	n.d.

Figure 15. Some of the most efficient tellurium based mammalian TrxR inhibitors. Despite strong inhibitory effects on TrxR-activity in *in vitro* assays, colony formation is not inhibited alike for all cell lines tested (MCF-7cells: human breast cancer cells; Colo320 cells and HT 29 cells: human colon cancer cells). Data taken from Ref. 324.

8. Naphthazarin Derivatives

Naphthazarin (Fig. 16) is known to display antiplasmodial and anticancer activity. It also inhibits human glutathione and thioredoxin reductase. Alkylation at carbon 2 and 3 abolished formation of glutathione conjugates and already increased the specificity for TrxR.[329] Bromination of the aromatic ring optimized the IC_{50}-values even further. Whereas unmodified naphthazarin is a competitive inhibitor, most of its new derivatives show—at almost stoichiometric concentrations—mixed types of inhibition.[330]

9. Arsenicals

Many arsenical compounds are known for their thiophilicity. Lin et al. could show that trivalent arsencials (far more than pentavalent compounds) are potent inhibitors of murine thioredoxin reductase.[331] The most effective compound tested was $CH_3As^{(III)}I_2$. A competitive ($K_i = 100$ nM) and a non-competitive component of inactivation was noted. The inhibition required NADPH, was time-dependent and—unlike the inhibition by gold(I)-compounds for example—not reversible with

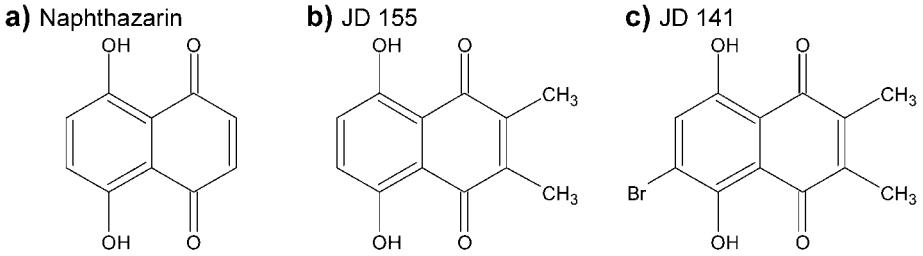


Figure 16. Naphthazarin (**a**) and its derivatives (**b**) JD 155 and (**c**) JD 141. Analysis of the inhibition of hTrxR-1 by these compounds (using DTNB as the substrate) resulted in IC_{50} values of 650 nM (a), 200 nM (b), and 5 nM (c), respectively. Similar results were obtained when using hTrx-1-Cys73 → Ser as the substrate. The results obtained with compounds of this series provided evidence that the bromination of the aromatic ring is responsible for this more than 100-fold increase in potency.[330]

chelating agents such as 2,3-dithiol succinic acid. The compounds were also effective in cell culture. However, a major drawback for these compounds as TrxR inhibitors is their lack of specificity: many other flavoenzymes such as glutathione reductase and small TrxRs are inhibited as well. Thus these reported effects may help us to understand the toxicity of arsenicals but appear not to be suitable as routine inhibitors of TrxR. It must furthermore be taken into account that the preparation of these compounds requires handling of highly toxic and carcinogenic substances.

10. Heavy Metal Ions

Thiols and selenols easily form complexes with heavy metal ions such as Hg^{2+}, Cu^{2+}, Zn^{2+}, Co^{2+}, and Mn^{2+}. The inhibitory effect of a given heavy metal ion correlates well with the solubility product of its inorganic sulfide.[210] However, this binding is non-specific, and thus heavy metal ions are suitable TrxR-inhibitors only in very specific experimental conditions.

11. Dicarboxylic Acids and Dithranol

Dithranol (= anthralin; Fig. 17A) and saturated dicarboxylic acids (Fig. 17B) with 6–12 chain carbon atoms have been reported by Schallreuter et al. to inhibit human TrxR in melanomas and psoriasis.[222,240] However, most data were obtained using the *E. coli* enzyme, a small TrxR. The experiments presented for the mammalian enzyme were mainly done on biopsies using an unconventional assay whose validity and specificity are questionable.[193,194] The published inhibitory effects of these compounds, especially the dicarboxylic acids, on human TrxR are minimal: millimolar concentrations were to be applied for almost 1 hr to yield inhibition. One must take furthermore into account that the commercially available purity of dicarboylic acids is approx. 99%. Thus, at a 10 mM concentration of the ''inhibitor'' up to micromolar concentrations of impurities may be present that do excert the inhibitory effect. Other authors, however, report an inhibition of 64% for rat liver TrxR using 100 μM azelaic acid[332]—i.e., higher than in Schallreuters original report. Furthermore, the authors state that this result was obtained after a preincubation in the *absence* of NADPH.

In our (and other) hands using highly purified hTrxR-1, no significant inhibitory effect was observed with these compounds, neither if added directly to the assay nor after preincubation (either in the presence or absence of NADPH).[210]

In their understandable attempt to find an inhibitor of human TrxR, Schallreuter and colleagues may have missed the importance of the inhibition of *E. coli* TrxR by azelaic acid, which they report to be much faster and can be observed at 10–100 times lower concentrations. If these experiments can be confirmed for other small TrxRs, these compounds may serve as lead structures for novel bacterio- and fungistatic agents based on TrxR-inhibition. Indeed earlier experiments using azelaic acid on bacterial cultures revealed an inhibition of DNA-synthesis.[239]

12. 13-Cis-Retinoic Acid

This compound (Fig. 17C) has been used for the treatment of *acne* and other skin diseases for many years. 13-*Cis*-retinoic acid, but not its isomer all-*trans*-retinoic acid, was first reported by Schallreuter

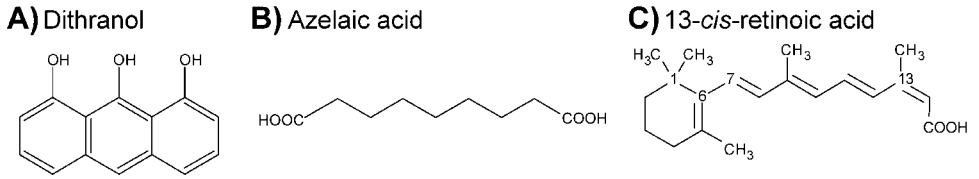


Figure 17. **A**: Dithranol. **B**: Azelaic acid, a dicarboxylic acid. **C**: 13-*Cis*-retinoic acid (= isotretinoin).

and Wood to covalently inhibit thioredoxin reductase.[333,334] 13-*Cis*-retinoic acid (1 mM) resulted in 30% decrease in activity. Similar results have later been published by Rigobello et al. who determined 15% inhibition at 100 μM and 35% at 500 μM.[181] U-Taniguchi et al. even report 86% inhibition at 1 μM after 30 min of preincubation in the absence of NADPH.[332] This finding is incompatible with the proposed mechanism which requires active site thiols.

Used in cell cultures, 86 μM 13-*cis*-retinoic acid was capable to reduce the reactivation of H_2O_2-damaged proteins—attributed to the action of the thioredoxin system—by 50%.[102]

The compound is, however, somewhat difficult to handle as it is for instance light sensitive and exhibits limited solubility.

13. Antitumor Quinoid Compounds

Several quinoids such as the quinones diaziquone, doxorubicin (= adriamycin), and menadione as well as the quinoneimine 2,6-dichloroindophenol (Fig. 18) have been reported to be effective inhibitors of rat TrxR *in vitro*, whereas others (e.g., actinomycine D and mitomycin C) had little or no effect.[207,209] The compounds showed a mixed type inhibition in the DTNB-reduction assay.[293] Initial competitive inhibition was observed with K_i-values of 7.5 μM (diaziquone), 10 μM (doxorubicin), and 4.2 μM (2,6-dichloroindophenol). After 1 hr of incubation in the presence of NADPH, the type of inhibition changed. Extensive dialysis could not reverse this inhibition indicating a covalent modification. As radioactive analogues could not be detected as labels of TrxR by SDS–PAGE analysis, an indirect modification was assumed. As the inhibition was more pronounced in the presence of oxygen and some ESR-spectra indicated the presence of radicals it may be speculated that oxidation of active site thiols and/or selenolates with the possibility of a consecutive loss of selenium (e.g., by β-elimination) contribute to the observed inhibition. Unfortunately, no attempts were made to reactivate the enzyme with reductants such as DTE. Our own attempts using doxorubicin and daunorubicin failed to result in a comparably effective inhibition of hTrxR-1[210] although we followed the protocol given in Ref. 209. Protective effects of hTrx-1 overexpression against doxorubicin cytotoxicity which were abolished by selenite and diamide treatment have been published.[278]

Taken together, the published quinoids cannot be recommended as TrxR inhibitors, especially not for *in vivo* trials where other effects of these compounds predominate.

9. SUMMARY AND OUTLOOK

The thioredoxin system is involved in a multitude of cellular functions in most species. Understanding the interactions of this system with other metabolic pathways and their physiological relevance are of

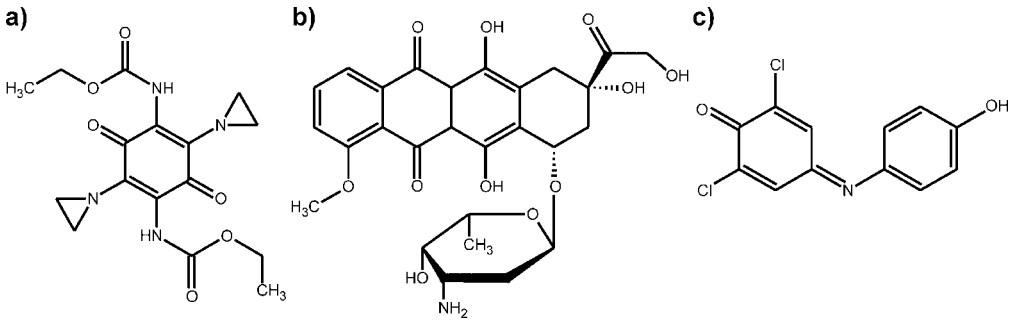


Figure 18. Quinoid compounds reported to be effective rTrxR inhibitors. **a**: Diaziquone; (**b**) doxorubicin (adriamycin); (**c**) 2,6-dichlorindophenol.

importance for a future rational therapeutic approach. Cancer, autoimmune diseases, and infectious diseases are presumably the most promising fields for Trx and TrxR inhibitors, yet there are other indications. Several highly selective and effective lead structures have already been discovered. One of the future aims is therefore to optimize these compounds by further increasing their bioavailability and selectivity and thus decreasing their unwanted side effects.

The results reported in this review justify the prediction that inhibitors of the thioredoxin system will become clinical standard drugs in several therapeutic fields in the future.

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