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Review

Thioredoxin system – a novel therapeutic target

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Abstract. Nowadays there are numerous pathogens that have created a resistance to commonly used antibiotics and drugs. Therefore research is focused on finding new therapeutic targets and on determination of their 3D structures that could help in designing new effective substances and inhibitors. Thioredoxin system not only plays a crucial role as thiol/disulfide redox controller, it is also essential for certain organisms as the only system ensuring the redox homeostasis. It is the redox-regulating function, which makes thioredoxin and thioredoxin reductase attractive for scientific research, especially in many studies of diseases caused by redox instability. Thanks to these facts, the proteins of thioredoxin system are suitable candidates for new therapeutic purposes. In this review we summarized the basic features of the thioredoxin system, we justified why the proteins of thioredoxin system are appropriate therapeutical targets and we provided overview of the possibilities of their inhibition by several types of inhibitors.

Key words: Thioredoxin system — Inhibitors — Oxidative stress

Thioredoxin system

Thioredoxin system (Trx system) was identified in organisms at all levels of evolution – from *archaea* to man. It is composed of proteins belonging to the oxidoreductase family: thioredoxin (Trx), an enzyme thioredoxin reductase (TrxR) and NADPH coenzyme. TrxR catalyzes electron transport from NADPH to the inactive – oxidized form of thioredoxin (Trx-S_2) and transforms it to an active, reduced form (Trx-$(SH)_2$) (Holmgren 1985). Reduced Trx can transfer reducing equivalents to many substrates such as insulin, coagulation factors, ribonuclease, choriongonadotropins and glucocorticoid receptor. Inside the cells Trx system acts also as a redox regulator, protects cells from damage caused by oxidative stress, scavenges ROS and controls a cellular redox balance. Trx system plays an important role in many physiological processes and it is also a prominent cell antioxidant (Kohárыová et al. 2008). For many organisms its presence is essential to life.

In higher organisms, the components of the Trx system are located in: nucleus, cytoplasm, chloroplasts, mitochondria, or they appears as extracellular or membrane-bound (Arnér and Holmgren 2000; Sőderberg et al. 2000; Watson and Jones 2003). Bacterial Trx system is located in the cytoplasm.

Thioredoxins (Trxs) are small thermostable proteins. Their common characteristic is a similar 3D structure, which consists of a core of five β-sheets surrounded by four α-helixes with a typical conserved active site sequence Cys-X-X-Cys, (two cysteines separated by two other amino acids). This small globular protein has 90% of its residues involved in secondary structure elements. It is an excellent model for computer modelling and theoretical analysis for investigating the relationship between the structure of proteins and their functional properties (Štefanková et al. 2005). All previously studied Trxs have 27 to 69% sequence identity with *E. coli* thioredoxin (Arnér and Holmgren 2000). Despite of their conserved structures, Trxs differ in reactivity, which can be caused only by slight variations in the main „fold".

The physiological functions of Trx in different organisms are diverse: from mainstream reactions where Trx acts as an electron carrier until to a large number of different highly specialized functions and reactions. Trx has a conserved role in the synthesis of DNA as a high-capacity hydrogen donor system for reducing the enzyme ribonucleotide reductase (Laurent et al. 1964), the Met-SO and PAPS reductase (Tsang

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and Schiff 1976; Ejiri et al. 1979). Thiol-redox control of Trx system also regulates chloroplast photosynthetic enzymes (Buchanan et al. 1994). However, Trx can modulate the activity of proteins through protein-protein binding to signal molecules, such as apoptosis signal-regulating kinase 1 (ASK1). Trx binds to the N-terminus and inhibits ASK1-kinase activity and thus also the ASK1-dependent apoptosis (Saitoh et al. 1998). Trx1-binding domain of ASK1 is a monomeric and rigid domain that forms a stable complex with reduced TRX1 with 1:1 molar stoichiometry (Kosek et al. 2014). By this mechanism *E. coli* Trx can increase processivity of T7 DNA polymerase and thereby affect the replication of phage T7 DNA (Huber et al. 1987).

In addition, Trx is able to interact with transcription factors and to undergo the posttranslational modifications such as: oxidation of the thiol groups of Cys-32 and -35, glutathionylation at Cys-73 (Casagrande et al. 2002), S-nitrosylation at Cys-69 or Cys-73 (Haendeler et al. 2002), nitrative modification at Tyr-49 (Tao et al. 2006) and also glycation (Yuan et al. 2010). This capability is also consistent with the role of Trx system in angiogenesis signaling. Trx1 in endothelial cells exerts proangiogenic effects throughout a broad range of cellular activities operating to angiogenic function including: transcription, posttranslational modification, migration, proliferation, vascular network formation, apoptosis and intracellular signaling (Dunn et al. 2010).

Highly specialized function exhibit chloroplast Trxs, which act as messengers of redox signals from ferredoxin to target enzymes. Moreover, *in vivo* experiment by Luo et al. demonstrated that one class of chloroplast Trx, named asTrx-M can compensate a lack of different class of chloroplast Trx, named as Trx-F. Both Trxs act as of important redox regulators of Mg-chelatase (Luo et al. 2012).

In recent years, the research is focused on the role of Trx against oxidative stress and its function in the control of programmed cell death and apoptosis (Arnér and Holmgren 2000). These functions depend on: the disulfide reductase activity of Trx, the amount of NADPH and from activity of TrxR.

Thioredoxin reductases (TrxRs) are dimeric enzymes belonging to the flavin-pyridine-nucleotide-disulfid oxidoreductase family that includes enzymes as lipoamide dehydrogenase and glutathione reductase (Williams 1992). TrxRs catalyze the transfer of two electrons from NADPH *via* FAD and N-terminal disulfide active site to the substrate. Bacterial TrxRs differ from human in many aspects. They have different: size, 3D structures, mechanism of catalysis and also position of active site (Luthman and Holmgren 1982). Unlike bacteria, mammalian TrxR (mTrxR) is very similar to glutathione reductase by the structure and function. In addition, mTrxR contains a C-terminal extended arm (about 16 amino acids in length) which includes another redox-active motif. This motif in mammals include selenium-containing amino acid selenocysteine (Sec) – an analogue of cysteine in which the sulfur is replaced by selenium (Gladyshev et al. 1996; Tamura and Stadtman 1996). It is assumed, that thanks to highly-reactive active site of mTrxRs, they display also functions not linked with function of Trx (Arnér and Holmgren 2000).

Mammalian cells also contain three isoforms of TrxR: cytosolic protein TrxR1, the mitochondrial protein TrxR2 and the testis specific thioredoxin glutathione reductase (TGR) (Sun 2005).

TrxR reduces not only oxidized Trx, but has a wide range of other substrates such as: non-disulfide substrate selenide, lipid hydroperoxides and H_2O_2 (Arnér and Holmgren 2000). The direct protein substrate of human TrxR is for example glutaredoxin 2, which can be also reduced by a glutathione (Johansson et al. 2004). Other substrates of TrxR are: cytosolic peptide granulysin (Björkhem-Bergman et al. 2004) and cytotoxic and antibacterial NK-lysine (Andersson et al. 1996). There are also various non-protein substrates for mTrxR, for example, regeneration of active vitamin C, when TrxR reduce dehydroascorbate to ascorbate (May et al. 1997), and also reduction of ubiquinone (Xia et al. 2003; Nalvarte et al. 2004) and cytochrome c (Nalvarte et al. 2004).

Recently it has been discovered that thioredoxin and glutathione systems of mammals have an ability to provide electrons reciprocally and serve as a backup system for each other. In contrast, bacteria TrxRs are low molecular weight enzymes with structure and reaction mechanism distinct from mammalian TrxR. Many bacterial species possess specific thiol-dependent antioxiadant systems, and the significance of the Trx system in the defense against oxidative stress is different. Bacterial, low molecular weight TrxR is also very specific and except for Trx, it accepts only a small amount of other substrates. Particularly, the absence of a GSH-Grx system in some pathogenic bacteria such as *Helicobacter pylori*, *Mycobacterium tuberculosis* and *Staphylococcus aureus* makes the bacterial system essential for survival under oxidative stress. This provides an opportunity to kill these bacteria by targeting the TrxR-Trx system (Lu and Holmgren 2014). In addition, differences between mammalian and bacterial TrxRs open up a new potential for designing new drugs or antibiotics.

Expression of thioredoxin system during oxidative stress in microbes

Despite the high structural similarity of proteins of Trx system, they are unique in their characteristics and function. Trx is an antioxidant protein, whose expression is enhanced by various forms of oxidative stresses. It is also known, that exercise-induced oxidative stress may affect Trx induction (Sumida et al. 2004). During oxidative stress, expression

of genes encoding proteins of Trx system is increasing and activities and location of proteins of Trx system proteins is changing considerably.

It was revealed, that expression of *Trx* induced by oxidative stress is accompanied by the binding of transcription factor Nrf2 (NF-E2-related factor 2) to the specific ARE element (antioxidant responsive element) present in the promotor region of *Trx* (Kim et al. 2001). Nrf2 is a redox-sensitive, leucine zipper transcription factor that was originally identified as a binding protein of the locus control region of β-globin gene (Moi et al. 1994). ARE is a cis-acting regulatory element or enhancer sequence, which was found in the promoter regions of genes encoding enzymes and proteins belonging to the phase II detoxification, which includes the proteins of Trx system (Lee and Johnson 2004).

In *E. coli* were identified two regulons directly responsive to H_2O_2-induced oxidative stress: transcriptional regulator OxyR, which binds to a target place and activates gene expression by contact with alpha subunit of DNA polymerase and regulon SoxRS. SoxR and SoxS serve as response regulators to the superoxide of enterobacteria (Christman et al. 1989; Tao et al. 1993; Nunoshiba 1996).

Although many gram-positive bacteria contain homologues of OxyR to control the expression of *Trx* genes, they use also other regulons such as sigma (σ) factors. σ factor is the specificity factor, which is additional subunit of RNA polymerase that directs RNA polymerase to the promoters of selective gene sets. Essential *trxA* gene in *B. subtilis* is controlled by σ^A factor and initiation of transcription is induced by H_2O_2. During continual stress, σ^B factor is also transcribed (Scharf et al. 1998).

In *S. coelicolor*, 65 σ factors of which 45 are ECF (extracytoplasmic function) σ factors, were identified. Previously described ECF σ factors in *S. coelicolor* respond to external stimuli and activate genes involved in disulphide stress, cell-wall homeostasis and aerial mycelium development (Paget et al. 2001). σ^R factor (25 kDa), encoded by *sigR* gene, has been identificated in *S. coelicolor* like a part of a system that senses and responds to thiol oxidation through the regulation of the transcription of many genes involved in the maintenance of thiol-disulphide redox homeostasis. The *trxBA* operon, encoding TrxR and Trx in *S. coelicolor*, was found to be under the direct control of σ^R. *trxBA* is transcribed from two promoters, *trxBp1* and *trxBp2*, separated by 5-6 bp. Paget et al. discovered, that exposure of *S. coelicolor* hyphae to oxidative stress causes intracellular thiol oxidation, which induces σ^R activity, leading to increased production of Trx and TrxR, as well as σ^R itself (Paget et al. 1998). σ^R factor binds to RsrA-binding domain under normal reducing conditions. RsrA (12 kDa) is a cognate anti-sigma factor, it is redox-sensitive zinc metalloprotein, which modulates σ^R activity. Exposure to disulphide stress induces the formation of one or more intramolecular disulphide bonds in RsrA, which causes it to lose afinity for σ^R, releasing σ^R to activate *trxBA* transcription (σ^R-dependent transcription). Increased *trxBA* expression, in turn, leads to the back thioredoxin-dependent reduction of oxidized RsrA back to its σ^R-dependent transcription (Kang et al. 1999). In addition, σ^R positively autoregulates the expression of the *sigR-rsrA* operon (Paget et al. 1998). Upon re-estabilishment of normal reduced thiol levels by the thioredoxin system, σ^R activity is switched off.

In all studied bacterial organisms, Trx and TrxR are expressed as two proteins except for *Mycobacterium leprae*, where Trx is expressed with TrxR from one gene. Result of expression is a hybrid protein with subunits linked by hydrophilic peptide linker (Wieles et al. 1995).

Expression of thioredoxin system proteins during oxidative stress in mammals

In pathophysiological conditions induced by oxidative stress was observed specific intracellular localization of Trx and TrxR. Although Trx1 have not localization sequence, it has been shown that a certain concentration of Trx1 is also present in the cell nucleus in normal physiological conditions and significantly increased after stimulation with oxidizing agents (Watson and Jones 2003). The translocation of Trx from the cytoplasm to the nucleus was first observed in cultures of keratinocytes after UV irradiation (Masutani et al. 1996). Translocation can be also caused by other reagents and conditions such as: hypoxia, exposure to H_2O_2, hemin, treatment of cells with PMA (phorbol 12-myristate 13-acetate) (Hirota et al. 1997; Ema et al. 1999). It is assumed that, the transfer of Trx is done by another protein with a nuclear import sequence, and this process can be influenced by caloric restriction (Cho et al. 2003). The presence of Trx1 has been also demonstrated in the extracellular space, into which reaches by unknown mechanism (Tanudji et al. 2003).

Trx in blood plasma even serves as a biomarker of oxidative stress (Nakamura et al. 1998; Nishinaka et al. 2001). Elevated plasma Trx was found in the development of a number of diseases, including hepatocellular carcinoma (Rubartelli et al. 1995), AIDS (Nakamura et al. 1996), Sjögren's syndrome (Saito et al. 1996) and rheumatoid arthritis (Yoshida et al. 1999).

High levels of antioxidant proteins, including increased expression of Trx were observed in cancer cells, which are often under high oxidative or hypoxic stress. Increased expression of Trx has been demonstrated in cells of many primary cancers including: cervical cancer (Cha et al. 2009), cancer of colorectal tract (Raffel et al. 2003), lung cancer (Kim et al. 2003), pancreas cancer (Han et al. 2002) and stomach cancer (Toddy et al. 2000). On the other hand, many studies suggest that overproduction of Trx plays not only active-protective role. Overproduction may promote

cancer proliferation in a number of ways such as: 1.) by direct stimulation of cancer progress, 2.) by inhibition of apoptosis in cancer cells, 3.) by stimulating the invasive and metastatic activity, but also 4.) by the creation of chemotherapy resistance in cancer cells induced by overproduction of Trx, which has been demonstrated for example in patients receiving cisplatin (Yokomizo et al. 1995; Sasada et al. 1996; Karlenius and Tonissen 2010). In early stage cancer patients, who are commonly treated by radiotherapy, Trx system was identified as a potential modulator of radiosensitivity (Smart et al. 2004; Mehta et al. 2009).

Thioredoxin system of selected pathogens as a therapeutic target

The cytoplasm of pathogens, like as in all organisms, is a highly reduced environment, in which cysteines of proteins are maintained in reduced state. But pathogens are also exposed to oxidative stress, which is produced by the metabolic pathways of aerobic pathogens themselves (reactive oxygen species) or it is produced by host organism as a defense against pathogens (reactive oxygen and nitrogen metabolites). During the increasing oxidative stress, pathogens activate different redox mechanisms to detoxify reactive oxygen metabolites, to reduce unwanted disulfide bonds and to repair damage in their cell.

In most cases, the organisms contain multiple Trx proteins. Their individual functions are still studying and revealing. Study is very difficult, because it indicates a complex network of antioxidant processes. Similarly as in many non-pathogenic organisms' genomes, also in pathogens have been identified a number of Trx and TrxR. For example, Trx system is a major thiol-disulfide redox system in gram-negative, opportunistic pathogen *B. fragilis* (Rocha et al. 2007) and contains six genes encoding homologues of Trx and one gene for TrxR (Reott et al. 2009). Recently studies revealed that not all Trx proteins are still active and providing an active protection. It is supposed that they can be a part of "redundant system", which ensures survival under special condition. It was found that one of three revealed thioredoxins in *M. tuberculosis,* TrxA has weak capacity to act as a disulfide reductase and it is not a substrate for TrxR under tested assay condition (Akif et al. 2008).

Pathogens lacking cooperating glutaredoxin-glutathione system have the Trx system as main redox system. Such organism is *H. pylori*, whose Trx system is composed of two Trx (Baker et al. 2001) and one TrxR (Gustafsson et al. 2007). Important physiological function was confirmed by the mutation experiments, in which mutations of Trx caused increased sensitivity *H. pylori* against to oxidative and nitrogen stress (Comtois et al. 2003).

Another example is the opportunistic pathogen *Bacteroides fragilis,* which belongs to the most tolerant obligate anaerobes to atmospheric oxygen and is able to survive a long time in a fully aerobic environment (Tally et al. 1975). This feature is allowed by large number of genes, which are expressed in aerobic conditions, including the genes for the Trx (Sund et al. 2008). *Entamoeba histolytica* affecting amoebic dysentery or amoebic liver abscess in humans and primates also belongs to the anaerobic parasites, which lack the glutathione system and an unusual form of glutathione-trypanothione (Ariyanayagam and Fairlamb 1999; Ryan and Ray 2004). For virulence factor of this organism is considered its ability to deal with the increased oxidative stress and with high concentration of reactive oxygen metabolites (Akbar et al. 2004). The main redox system is Trx system with peroxiredoxin and cofactor NADPH, which can successfully reduce hydroperoxides (Arias et al. 2007).

A role of Trx system against oxidative stress is also important for mycobacteria that resides in the host organism in macrophages, what presents form of virulence. Macrophages, neutrophils, and other fagocyting cells are key antimicrobial components of the immune response, due to the fact that they can generate a large amount of highly toxic molecules as reactive oxygen and nitrogen metabolites, and hydrolytic enzymes such as acid hydroxylase (Chan et al. 1992; Clemens and Horwitz 1995; Rich et al. 1997). Despite this toxic environment *M. tuberculosis* can survive thanks to several mechanisms (Pieters, 2008). Mycobacteria and other actinomycetes (e.g. *S. coelicolor*) do not synthesize glutathione but in cells they have mycothiol (Newton et al. 1996) and their redox regulation is also under control of Trx system. In mycobacteria, it has been confirmed that mycothiol plays important role in detoxification, quenching reactive oxygen and nitrogen metabolites formed in phagocytes during infection and provides reducing equivalents for peroxiredoxins (Shinnick et al. 1995; Zhang et al. 1999; Jaeger et al. 2004). In this way, Trx system is essential for pathogens and helps them survive in host cells. In 2008, TrxR *M. tuberculosis* was selected from nearly 4000 gene products, together with 541 other proteins as promising therapeutic target for the development of new antituberculotics based on bioinformatic predictions (Raman et al. 2008).

Thank to the clear differences in the properties of TrxR from bacteria and higher organisms (such as size, substrate specificity, structure and mechanism of catalysis) TrxR had just become a new promising therapeutic target for the preparation of new antibiotics (Gromer et al. 1998a; Becker et al. 2000; Hirt et al. 2000). In many cases, TrxR is essential enzyme for the physiology of the organism and deletion or inactivation of *TrxR* encoding gene may cause lethality, such as in the most causative agent of staph infections – gram-positive bacterium *Staphylococcus aureus* (Uziel et al. 2004).

TrxR from *P. falciparum* was identified as a promising target for the development of new antimalarial drugs (Banerjee et al. 2009). Using high-throughput screening program, specific inhibitors were identified allowing study of TrxR in relation to survival of the parasites (Williams et al. 2000).

The current trend in novel drug development is rational design of inhibitors, based on 3D structures of therapeutical targets. For this purpose, it has been crystallized and solved 3D structures of TrxR from gastric pathogen *H. pylori* (Gustafsson et al. 2007) and *M. tuberculosis* (Akif et al. 2005).

Importance of thioredoxin system in medicine

Thanks to explosive growth of studies focused on oxidative stress and oxidative damage, it has been found that a number of completely different diseases (such as infarct, stroke, lung cancer, cataracts, diabetes, and others) have a similar reason (Young et al. 2004; Schweizer et al. 2004; Varsik et al. 2006). The key role in their genesis plays a free radicals and oxidative stress, which affect formation of undesirable disulfides. After recording of oxidative stress, changes in the concentration and activity of proteins of Trx system are displayed. Especially, in comparison with normal tissues, many primary tumors overproduce proteins of Trx system, which helps to increase the proliferation of malignant cells (Powis et al. 2000).

The cytoplasm and mitochondria contain equivalent Trx systems and inhibition of either system can lead to activation of apoptotic signaling pathways. There are a number of inhibitors with chemotherapy applications that target either Trx or TrxR to induce appoptosis in cancer cells. Suberoylanilide hydroxamic acid (SAHA) is effective against many cancer cells and functions by up-regulating an endogenous inhibitor of Trx. Other compounds target the selenocysteine-containing active site of TrxR. These include gold compounds, platinum compounds, arsenic trioxide, motexafin gadolinium, nitrous compounds and various flavonoids. Inhibition of TrxR leads to an accumulation of oxidized Trx resulting in cellular conditions that promote apoptosis. In addition, some compounds also convert TrxR to a ROS generating enzyme (Tonissen and Di Trapani 2009).

Mammalian TrxR isoforms in the cytosol and the mitochondria are essential selenoenzymes with a selenocysteine in the active site. These enzymes display remarkably broad substrate specificity but are also targets for existing chemotherapeutic drugs. Mammalian TrxR enzymes are linked to selenium metabolism as a result of being selenoproteins, but can also directly reduce low molecular selenium compounds like selenite and have been implicated in the chemoprevention effects of selenium against cancer. Numerous scientific reports describe higher expression of Trx and TrxR in some, but not all tumours. Some data suggest that high Trx could be linked to resistance to chemotherapies while others suggest that high Trx and TrxR may induce apoptosis and reduce the mitotic index of certain tumors linked to the p53 dependent cell death. Recent data suggest that TrxR is essential for the carcinogenic process and invasive phenotype of cancer. Both Trx and TrxR have been regarded as interesting targets for chemotherapy (Arnér and Holmgren 2006).

The difficulty of delivering clinically relevant doses to kill all tumour cells in patients with lung cancer remains a true clinical problem. This is partly due to the organs at risk, i.e. normal lung and spinal cord that limit the total dose of irradiation that can be delivered to the tumour. The thioredoxin system is promising target when aiming to overcome the problem of clinical radiation resistance. Altered cellular redox status and redox sensitive thiols contributing to induction of resistance strongly connect the ubiquitous redox enzyme TrxR to the cellular response to ionizing radiation.

The study reveals increased radiation sensitivity of cultured lung cancer cells [cell line U1810] with inhibition of the multi-functional redox enzyme TrxR by the phosphine gold(I) complex [Au(SCN)(PEt3)] demonstrated by a decreased ability to recover after radiation treatment. The data clearly demonstrate an important role of TrxR in radiation resistance, and warrant further studies to elucidate the specific mechanisms of TrxR involvement in resistance development. Pharmacological inhibition of TrxR is an attractive treatment strategy to optimize the efficacy of radiation therapy and TrxR inhibition (Selenius et al. 2012).

Inhibitors of Trx

By several biological screenings were already detected different, small organic compounds which inhibit the Trx system.

Effective inhibitors of Trx-1/TrxR in MCF-7 human breast cancer with an IC_{50} 31–37 μM are alkyl 2-imidazolyl disulfides (Powis et al. 1998). Growth inhibition of two breast tumor cell lines was founded by Palmarumycin CP_1 with manifested IC_{50} 1 μM and 2.4 μM. This inhibitor is a natural fungal metabolite belonging to the family of napthoquinone spiroketal compounds with a direct effect on Trx (Wipf et al. 2001).

Inhibitor PX-12 (1-methylhydroxypropyl 2-imidazolyl disulfide) causes inhibition of Trx-dependent cell growth (Wipf et al. 2001) by irreversible thiolalkylation of Cys73 in Trx-1 and its antitumor activity was demonstrated against human tumor xenografts in *scid* mice (Kirkpatrick et al.

1998). The median IC_{50} for inhibition of several tumor cell-lines is 8.1 μM (Powis and Montfort 2001).

Para-quinone NSC401005, natural product of pleurotin was discovered by COMPARE analysis. IC_{50} of this compound against Trx-1/TrxR was determined as 0.17 μM, but using this inhibitor for testing on other types of tumor cells, the average IC_{50} was only 21.5 μM (Kunkel et al. 1997; Patent application US 20020049221 A1).

Compound pleutropin NSC-131233 together with PX-12 reduced the activity of the transcription factor HIF-1α (which responds to hypoxia) and also the expression of downstream targets of HIF-1, VEGF and iNOS *in vitro* (Welsh et al. 2003).

AW464 (4-hydroxy-4-(bezothiazol-2-yl)cyclohexadienone) is effective against renal- and colon-cancer cell lines, where inhibits Trx redox cycling by forming an irreversible complex with the active-site thiol groups in the reduced Trx (Pallis et al. 2003). AW464 has anti-proliferative activity on tumor cell lines and endothelial cells *in vitro*, but not fibroblasts, with IC_{50} value of 0.5 μM (Mukherjee et al. 2005).

MOL294 (methyl (4R/S)-4-hydroxy-4-[((5S,8S)/(5R,8R))-8-methyl-1,2-dioxo-2-phenyl-2,3,5,8-tetrahydro-1H-[1,2,4]triazolo[1,2-a]pyridazin-5-yl]2-butynoate) is a small organic molecule, which is designed to mimic the extended (β) strand of peptide substrates that bind Trx. MOL 294 inhibits NF-kB-mediated expression of vascular cell adhesion molecule 1 with IC_{50} of 2.5 μM *in vitro* (Misra-Press et al. 2002).

Inhibitors of TrxR

Recent studies have elucidated that TrxR was upregulated in many malignant tumors and inhibition of TrxR could prevent the tumor initiation and progression, suggesting TrxR to be a promising target for cancer therapy and the highly nucleophilic and accessible selenocysteine (Sec) active site might be the prime target for drug design. Various kinds of TrxR inhibitors have been developed as anticancer agents for years. TrxR inhibitors are divided into three classes, including metal containing inhibitors, naturally occurring products and their derivates and other newly emerged inhibitors (Yang et al. 2012).

Mammalian TrxRs have wide substrate specificity, because in addition to N-terminal redox active disulfide site "-Cys-Val-Asn-Val-Gly-Cys-" contain also secondary redox centre in the C-terminal with a typical "-Gly-Cys-Sec-Gly-COOH" sequence. This sequence is essential for catalysis and easily accessible for the different substrates and inhibitors (Fujiwara et al. 2001). Summary of IC_{50} of selected inhibitors are in Table 1.

Auranofin (AF)

Selenocysteine (Sec) group in the active site of reduced TrxR shows high reactivity towards metal ions (Witte et al. 2005). Organic gold compounds such as auranofin (2,3,4,6-tetra-O-acetyl-1-thio-β-D-glucopyranosato-S-(triethylphosphine) gold) exert cytotoxic effects by causing direct mitochondrial

Table 1. Overview of selected thioredoxin reductase (TrxR) inhibitors

Inhibitor	Description	Source of TrxR	IC_{50} (μM)	References
Myricetin	Flavonol	rat	0.62	Lu et al. 2006
Quercetin			0.97	
Catechin			6.6	
Pelargonidin	Anthocyanidin		4.2	
Taxifolin	Flavanone		6.2	
$HgCl_2$	Mercury compound		0.0072	Carvalho et al. 2008
Methylmercury (MeHg)			0.0197	Wagner et al. 2010
Motexafin gadolinium	Gadolinium-containing compound		6	Hashemy et al. 2006
Benzene-sulfonyl-6F-indole-substitued quinol	Quinol analog		4.3	Chew et al. 2008
Auranofin	Gold-containing molecule	human placenta	0.02	Gromer et al. 1998b
Auxil		human cancer cells	0.21	Rigobello et al. 2004
Hypericin	Naphthodianthrone	cytosolic rat liver	157.08	Sorrentino et al. 2011
		mitochondrial rat liver	43.12	
Pseudohypericin		cytosolic rat liver	4.4	
		mitochondrial rat liver	7.45	

damage through selective modification of the selenol active site in TrxR (Rigobello et al. 2004) and induces mitochondrial permeability, which results in the release of cytochrome c from mitochondria into cytoplasm with consequent apoptotic cell death (Cox et al. 2008). AF-like gold complexes synthetized by Gandin et al. are super inhibitors of TrxR1 and TrxR2 with IC_{50} values with low to sub-nanomolar range and also inhibits cancer cell growth (Gandin et al. 2010). AF is probably the most effective inhibitor of mammalian TrxR found to date and might be utilized as an anticancer agent to induce apoptotic cell death.

To a first approximation, TrxR inhibition may be attributed to binding of Au to the C-terminal redox-active –Gly-Cys-Sec-Gly sequence, as recently suggested by MALDI-TOF experiments. However, selenols and selenides have higher affinity for Au than thiols for several factors: 1. the general afinity between heavy polarizable atoms, 2. the consequent higher nucleophilic strenght of Se than S, 3. the lower pKa of selenols (5.2) than thiols (8.0), whereby the –SeH group of the Sec residue is completely dissociated at physiological pH, which makes –Se– a better nucleophile than the undisociated thiol (Di Sarra et al. 2013).

Arsenic trioxide (ATO)

ATO has been used as an anticancer drug for several thousands of years in traditional medicine and recently has been shown as an effective cancer therapeutic drug for acute promyelocytic leukemia and has potential anticancer activity against a wide range of solid tumors. ATO exerts its efect mainly through elevated oxidative stress, but the exact molecular mechanism remains elusive. Lu et al. discovered that ATO irreversibly inhibits mammalian Trx with an IC_{50} of 0.25 μM. Both the N-terminal redox-active dithiol and the C-terminal selenothiol-active site of reduced TrxR may participate in the reaction with ATO. The inhibition of MCF-7 cell growth by ATO was correlated with irreversible inactivation of TrxR, which subsequently led to Trx oxidation. The inhibition of TrxR by ATO was attenuated by GSH, and GSH depletion by buthionine sulfoximine enhanced ATO-induced cell death. These results strongly suggest that the ATO anticancer activity is by means of a Trx system-mediated apoptosis. Blocking cancer cell DNA replication and repair and induction of oxidative stress by the inhibition of both Trx and GSH systems are suggested as cancer chemotherapeutic strategies (Lu et al. 2007).

Cis platin

Thanks to strong nucleophilicity of Cys/Sec residue in TrxR it was supposed the reaction with potential electrophilic molecule – cisplatin (cis-diamminedichloroplatinum (II)). Sasada et al. showed that cisplatin inhibits isolated cellular TrxR with celullar enzyme activity to ~50% at a concetration of 10 μM (Sasada et al. 1999).

Gold-based compounds are essentially ultilized for the treatment of rheumatoid arthritis and they have also been tested for other pathologies such as AIDS, bronchial asthma, malaria and together with platinum-containing complexes can be also used as cancer chemotherapeutic drugs (Mirabelli et al. 1986; Shaw 1999).

Curcumin

Another type of inhibitor – curcumin, is yellow lipid-soluble polyphenol from the plant *Curcuma longa*. Curcumin is a part of food additive turmeric, in which 3–4% of weight is consisted from yellow pigments curcuminoids. It is a mixture of curcumin (94%), demethoxy curcumin (6%) and bis- demethoxy curcumin (0.3%). It has been shown that curcumin is a potent inhibitor of tumor initiation *in vivo* and also *in vitro* (Aggawarl et al. 2003). Later, it was found that curcumin is an antioxidant with anticancer activity and can irreversibly inhibit TrxR by simultaneously elicited an NADPH oxidase activity of the enzyme (Fang et al. 2005). Following these discovery, explosive number of synthetic analogs of curcumin with IC_{50} values from the nano- to the low micro- molar range was prepared and tested (Liu et al. 2008; Zhong et al. 2008). Very interesting are the recent studies which shown, that curcumin can turn to be a prooxidant to produce ROS and induce oxidative stress in cells (Han et al. 2011; Kuo et al. 2011). Cai et al. revealed, that curcumin modified TrxR1 oxidizes NADPH to generate superoxides *in vitro*, and curcumin-treated HeLa cells have elevated intracellular ROS production and curcumin can also drastically down-regulate Trx1 level as well as its enzyme activity in HeLa cells (Cai et al. 2012).

Flavonoids

Another promising group of TrxR inhibitors are flavonoids – products of secondary metabolism with widespread biological properties such as: antioxidant, antiproliferative, anti-inflammatory or antibiotic activity, which can contribute to their chemoprevention for the development of cancer and cardiovascular disease (Yang et al. 2001; Ross and Kasum 2002). In some cases, they exhibit pro-oxidant properties (Williams et al. 2004). Lu et al. tested flavonoides from all six categorized group to inhibition of mammalian TrxR and found out, that 3-hydroxyl-containing flavonoids such as quercetin, myricetin, taxifolin, catechin and pelargonidin exhibited an NADPH-, concentration- and time-dependent inhibitory effect (Lu et al. 2006).

It was investigated the inhibition of mammalian TrxR by flavonoids which have been presumed to be cancer chemoprevention agents because of their antioxidant activities.

Myricetin and quercetin were found to have strong inhibitory effects on mammalian TrxRs with IC_{50} values of 0.62 and 0.97 μM, respectively. The inhibition was shown to be concentration-, NADPH- and time-dependent and involved an attack on the reduced COOH-terminal of -Cys-Sec-Gly active site of TrxR. Oxygen-derived superoxide anions enhanced the inhibitory effect whereas anaerobic conditions attenuated inhibition. Spectral analysis suggested that the flavonoids might perform their inhibitory effects *via* semiquinone radicals. Additionaly, the flavonols had the potential to inhibit the growth of A549 cells with the same potency as inhibition of TrxR. TrxR activity in the cell lysates was reduced on treatment with myricetin >50 μM, which coincided with the oxidation of Trx. The cell cycle was arrested in S phase by quercetin and accumulation of cells in sub-G1 was observed in response to myricetin. Thus, the anticancer activity of quercetin and myricetin may be due to inhibition of TrxR, consequently inducing cell death (Lu et al. 2006).

Bacterial TrxR do not have secondary active site with highly reactive Sec group and this is probably one of the reasons why many mammalian TrxR inhibitors are without effect. Example is benzenesulfonyl-6F-indole-substitued quinol that selectively inhibits mammalian TrxR but is without effect on bacterial TrxR and glutathione reductase (Chew et al. 2008).

Ebselen

Selenoorganic compound ebselene [2-phenyl-1,2-benzisoselenazol-3(2H)-one] affects bacterial and mammalian TrxR *via* different mechanisms. Ebselene is an antioxidant and anti-inflammatory selenoorganic compound used in clinical trials against e.g. stroke (Wójtowicz et al. 2004). Mechanism of its action is mainly through its interactions with the mammalian TrxR and Trx providing the electrons for reduction of hydrogen peroxide. Holmgren et al. discovered that ebselene is not a substrate of *E. coli* TrxR but instead it is a competitive inhibitor for the reduction of Trx with K_i of 0.15 μM. Also *E. coli* mutants lacking glutahione reductase and glutathione are much more sensitive to inhibition by ebselene (Holmgren et al. 2011).

Conclusion

Proteins of thioredoxin system play essential regulatory role in all living organisms thanks to their huge functional diversity. The number of studies that reveal new physiological as well as pathological mechanisms in which these proteins play irreplaceable role is still increasing.

Trx and TrxR are interesting targets for therapeutic usage, and at present there is a rapid development of potential inhibitors, which are used not only in cancer therapy, but also in the development of new drugs against pathogens.

One of the best ways to create or design the effective inhibitor is using of compounds, which are designed after determination of protein structure of revealed mechanism of action.

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